ANNALS OF TROPICAL MEDICINE
AND PARASITOLOGY

THE UNIVERSITY OF LIVERPOOL

ANNALS

OF

TROPICAL MEDICINE AND

PARASITOLOGY

ISSUED BY THE

LIVERPOOL SCHOOL OF TROPICAL MEDICINE

Edited by

PROFESSOR WARRINGTON YORKE, M.D. M.R.C.P.
PROFESSOR D. B. BLACKLOCK, M.D.
PROFESSOR W. S. PATTON, M.B.
PROFESSOR R. M. GORDON, M.D.

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Robt. Newstead

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(2) Two courses for the Diploma in Tropical Hygiene, commencing on the 23rd April, 1930, and the 12th January, 1931. The D.T.H. examinations are held in March and July.

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ANNALS OF TROPICAL MEDICINE AND PARASITOLOGY

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Articles for publication should not exceed twenty-five pages of the Annals, and will be understood to be offered alone to this Journal. They should be typewritten and addressed to :—The Editors, School of Tropical Medicine, The University, Liverpool.

Illustrations for text figures or charts should be drawn clearly and firmly in Indian ink, if possible on Bristol board. N.B.—*Blue or other coloured* ruling in squares or lines cannot be reproduced.

All lettering, names or legends on text-figures, charts or maps should be printed *sufficiently large* to allow of *clear legibility* on reduction if necessary.

Plates and illustrations should be accompanied by short explanations.

References to authors in the text must be made in the following way :—' According to Smith (1900) the spleen is enlarged, but Robinson (1914) says the reverse.' The references should be collected in alphabetical order of authors' surnames at the end of the paper, and arranged in the following way :—

ROBINSON, S. (1914). The spleen in malaria. *Ann. of Nosology,* **20,** 20-25.

SMITH, J. (1900). Enlargement of the spleen in malaria. *Jl. of Pathometry,* **1,** 1-20.

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ON A NEW SPECIES OF
EIMERIA (*E. SOUTHWELLI*) FROM
AËTOBATIS NARINARI

BY

AHMED HALAWANI, Dr. med. (Würzburg), D.T.M., D.T.H.

(*From the Department of Parasitology, Liverpool School of Tropical Medicine*)

The oöcysts of the new species of *Eimeria* described below were found in the spiral valve of two specimens of intra-uterine embryos of *Aëtobatis narinari*, caught on the Ceylon Pearl Banks (9 October, 1920). These specimens were kindly given to me for investigation by Dr. T. Southwell, F.R.S.E.

The oöcysts, which were found in large numbers, are of various shapes, i.e., they are polymorphic. The immature ones have, in most cases, a pear-shaped appearance. During development they gradually become more and more elongated and uniform in diameter. The bulbous end of the pear decreases at each stage of development ; the great majority of the mature oöcysts are cylindrical bodies with rounded extremities. Only in a very few cases does the oöcyst, when it contains four sporoblasts, retain the bulb, and then only to a slight degree. Small spherical immature oöcysts were occasionally seen.

Measurement of the oöcyst. The length of the oöcyst in its various stages ranges from 15μ to 50μ, but these two extremes are rather rare. The average length is about 30μ and the breadth 12μ. The pear-shaped oöcysts have a diameter of about 20μ at the broad end and from 10μ to 12μ at the narrow extremity. The mature oöcyst ranges in size from 25μ to 50μ ; its average length is 38μ and its breadth 12μ.

The oöcyst is colourless and transparent ; its wall shows a double contour, and has a thickness of about 2μ.

The immature oöcyst, in most cases, contains the zygote as an unsegmented spherical body situated in the bulbar extremity, immediately beneath the cyst wall. There are usually from one to three spherical refractile bodies, each of which has an average diameter of 7μ or 8μ A residual mass is usually present in the immature oöcyst. It often disappears from the oöcyst as the latter matures.

Fig. 1. *Eimeria southwelli* n.sp. a.—An immature pear-shaped oocyst containing a large spherical zygote and a refractile body ; b.—An immature oocyst retaining its hind bulb to a slight degree ; c.—an oocyst in which the cytoplasm is dividing   d. to g.—Oocysts which are more or less cylindrical and each of which contains four sporoblasts ; h.—A fully-mature oocyst ; the sporocysts are oval in shape ; i.—An immature oocyst as seen in optic section.

As mentioned above, in the great majority of cases the mature oöcyst is cylindrical, but in other cases it may be sausage-shaped. The sporoblasts are arranged lengthwise, end to end, sometimes in pairs and sometimes in a chain. The young sporoblasts are at first conical but later on become almost spherical; occasionally they contain small, refractile, residual bodies. The sporocysts are oval and measure from 10μ to 12μ in length.

A micropyle could not be seen, but in one immature oöcyst a small narrowing of the oöcystic wall appeared to indicate its position at one end.

In order to investigate the extent of the infection in the tissues of the host, a series of sections of the liver and gut were stained with Heidenhain's Iron Haematoxylin and Meyer's Haemalum. The

two intra-uterine embryos submitted for examination had been preserved intact in formalin since 9 October, 1920.

Microscopically the whole of the liver structure was completely broken down. It could not be decided whether this was due to massive infection with the parasite or to bad preservation of the tissue. Most probably it was due to both. The epithelium of the bile ducts and the liver cells were destroyed to such an extent that it was difficult to make out their structure definitely. In some parts collections of immature oöcysts and cell debris were found in small cavities.

The same picture of cellular disintegration as presented by the liver was also seen in sections of the gut. The greater part of the tissue was destroyed and this was associated with the presence of oöcysts, some of which were embedded in the tissue.

As far as I am aware no species of the genus *Eimeria* has been recorded previously from this host in any locality, and only two species have hitherto been obtained from Elasmobranch fishes, namely :

1. *E. lucida* Labbe, 1893 ; from *Mustellus canis, Scyllium stellare*, and *Acanthias acanthias* (oöcysts 10μ to 11μ).

2. *E. gigantea* (?) Labbe, 1896 ; from *Lamna cornubica* (oöcysts 70μ).

As the oöcyst in our species differs in size and morphology from the above two, I therefore consider that it is a new parasite, for which I propose the name *Eimeria southwelli*.

How the infection is acquired by the intra-uterine embryos is an interesting matter. Most probably the oöcysts, which develop to maturity inside the host in the case of cold-blooded animals, were conveyed from the mother to the embryos via the cloaca, vagina, and uterus of the former.

I must express here my indebtedness to Dr. T. Southwell, F.R.S.E., for presenting me with the material, and for his valuable help and criticism.

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WENYON, C. M. (1926). Protozoology. A manual for medical men, veterinarians and zoologists. London.

METAZOAN IMMUNITY : A REPORT ON RECENT INVESTIGATIONS*

BY

D. B. BLACKLOCK

R. M. GORDON

AND

J. FINE

WITH A BACTERIOLOGICAL INVESTIGATION

BY

MARION WATSON

I. INTRODUCTION

In a previous paper, Blacklock and Gordon (1927) gave an account of the experimental production in guinea-pigs of immunity to an insect parasite, *Cordylobia anthropophaga*. Since the publication of that paper the existence of a definite immunity to certain parasites belonging to the metazoa has been recognised and interesting results have been published. For example, Sandground (1928), who carried out extensive experimental investigation with the nematode parasite *Strongyloides stercoralis*, has demonstrated that immunity against this parasite can be acquired. Similarly, Stoll (1928) has shown that there can occur an immunity on the part of sheep against another nematode parasite, *Haemonchus contortus*. Both authors are dealing with an immunity acquired as a result of infections produced experimentally through the natural channels. In the paper on Cordylobia referred to above, there were recorded some experiments on attempts to produce artificial immunity by the application in various ways of larvae or extracts of larvae ; of

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these experiments a few were successful in producing immunity in the areas treated. Since that time we have performed numerous experiments involving the treatment of several hundred guinea-pigs and rats, with the aim of discovering some simple and readily applicable method of producing the immunity by artificial means. Over forty animals have been treated for more or less prolonged periods by injection of antigen prepared from (i) First instar larvae, and (ii) Third instar larvae—(a) Salivary glands, (b) Gut, (c) Cuticle, (d) Haemocoele fluid, (e) Excreta. Of the forty animals treated, twenty-three survived throughout the course of the injections and the subsequent incubation and observation periods ; of these twenty-three animals only three developed complete immunity of the areas of skin treated.

The amounts and methods of administration of the antigen required to produce the immunity in the case of these animals were as follows :—

1. ANIMAL No. 127. Received by intracutaneous injection 230 first instar larvae in 5 injections extending over a period of 32 days.

2. ANIMAL No. 129. Received by intracutaneous injection the ground-up cuticle of 42 third instar larvae in 6 injections, extending over a period of 30 days.

3. ANIMAL No. 130. Received by intracutaneous injection 521 first instar larvae in 5 injections extending over a period of 28 days.

Repeated controls, using for similar injections equal quantities of the vehicle employed, i.e., normal saline or 50 per cent. glycerine, proved ineffective, the normal number of larvae developing on animals treated with such injections. It should be mentioned that although only three animals were rendered completely immune by injections, it was observed that many of the animals showed a relative immunity after treatment by injection of antigen.

At the same time that the series of injections designed to produce artificial immunity was being carried out, numerous animals were rendered immune by the process of allowing larvae to penetrate the skin and develop. The results of long series of such applications of larvae conformed exactly to our previous findings, complete immunity being produced with almost unfailing regularity ; with equal

regularity the control animals permitted the full development of the usual proportion of larvae.

Guinea-pigs of local origin, as we pointed out previously, enjoy a relative immunity against Cordylobia. We recorded that whereas 59 per cent. of larvae survived to the sixth day on imported English guinea-pigs, only 33 per cent. of larvae survived in local guinea-pigs for this period. We considered it advisable to continue the observation of imported and local guinea-pigs as this difference in the susceptibility of imported and local guinea-pigs doubtless bears some analogy to the partial immunity, frequently referred to in the literature, against hypoderma in endemic areas. The figures most recently obtained confirm our previous findings. Thus, of 215 first instar larvae placed on imported English guinea-pigs, 122, that is 57 per cent., survived the observation period of 6 days, whereas of 413 larvae placed on local guinea-pigs, only 120, that is 29 per cent., survived. We concluded in our previous paper that this relative immunity was due to previous infection of local guinea-pigs by Cordylobia larvae, but we were not at that time successful in demonstrating the truth of this theory by any laboratory tests. Since then, however, we have discovered that a precipitinogen which reacts with the serum of previously infected animals is present in the haemocoele fluid and the excreta of the third instar larva; this precipitin test differentiates sharply animals which have been infected previously from animals which have not been so infected. The application of this test has demonstrated that the relative immunity of local races of guinea-pigs is due to a former infection with Cordylobia.

The discrepancy between the results obtained by the application of small numbers of living larvae on the one hand, and the injection of large amounts of dead larval antigen on the other, suggested strongly that the living larva during its existence as a parasite in the host supplies the antigenic substance necessary to immunity in a more potent form or in greater amount than we do by the injection of any save quite impracticable quantities of antigen prepared from dead larvae or portions of larvae.

The situation at this time, therefore, was that an immunity, readily enough produced by imitating closely the natural mode of infection, could be produced only exceptionally by artificial means.

Not only so but the antigens, even in their most concentrated form, represented a large quantity of injection material, the preparation of which involved for each animal treated a quite uneconomic expenditure in time and labour.

Failing to obtain satisfactory artificial immunity by the vaccination of animals with crude and bulky material, we undertook a more detailed biological study of the larva in its various instars in the hope that this would eventually lead us to an understanding of the principles upon which the immunity depends. The methods used and results obtained are detailed below.

II. TECHNIQUE

Dissection of the larva.

All dissections were carried out with the aid of a dissecting microscope, even in the case of large third instar larvae. Owing to the close relationship of the salivary glands and portions of the malpighian tubes, and also to the denseness of the branching of the tracheal system adhering to them, it is difficult to extract the salivary glands intact without a somewhat elaborate dissection. Two circular incisions are made round the entire larva, one at the cephalic end and the other as close to the anus as possible. These circular incisions are connected by a ventral incision and the flaps reflected. The salivary glands having been removed all the other portions of the larva can be easily isolated. In the case of first instar larvae it is as a rule sufficient to nick the cuticle on both sides in the region of the third segment, using very fine needles (e.g., Kirby, No. 14) and then to exert traction on the cephalic portion of the larva with one needle while fixing the caudal portion with the other ; by this method the salivary glands generally come out entire attached to the cephalic extremity.

Method of obtaining the haemocoele fluid.

In order to open the haemocoele cavity without injury to the gut and other internal organs, two spatulate needles were employed to press away the gut from the body wall ; an incision with a sharp knife was then made between the needles through both layers of the skin (fig. 1). When the incision is made with these precautions,

not only is the gut uninjured, but also the flow of haemocoele fluid from the cut can be very accurately controlled. The fluid does not escape through the incision until the pressure of the two flat needles is released, and according to the degree of relaxation of the pressure the flow can be graduated.

FIG. 1.
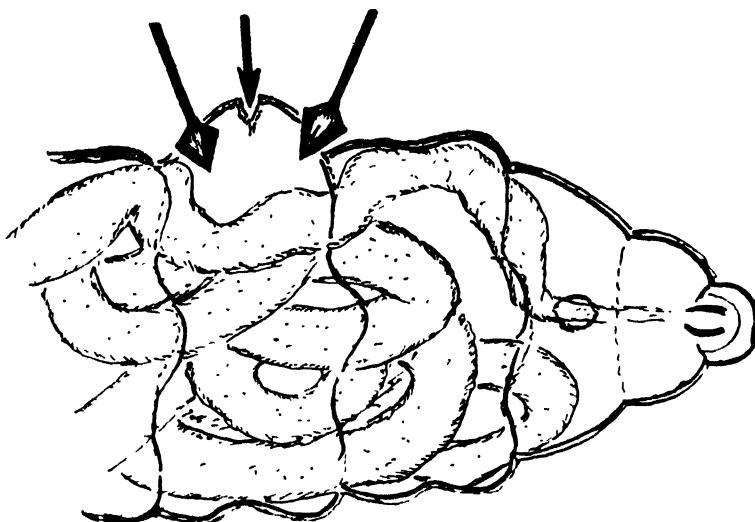
If it is desired to gain access to any special portion of the gut, the haemocoele cavity is incised and tapped in the position corresponding to the section of the gut required, and after the haemocoele fluid has drained off, the gut is allowed to prolapse through the incision.

Sterile dissection of larva.

The exterior of the larva was sterilized when it was necessary to obtain haemocoele fluid or gut free of outside contamination. Using the technique described above, the area of larval skin stretched between the two needles was seared above and below with a hot blade. After the blade had passed between the upper surface of the slide and the under surface of the larva, the latter was made to adhere to the slide by pressure of the hot blade. The incision could then be made with aseptic precautions and haemocoele fluid or gut obtained with minimal danger of external contamination.

In cases where more extensive aseptic dissection was necessary, the entire larva was washed well in running water, immersed for half-an-hour in a 2 per cent. aqueous solution of perchloride of mercury, washed thrice in sterile normal saline solution and dissected with aseptic precautions on a sterile slide. The last saline used for washing was tested for sterility ; it was also tested for the presence of mercury, using Nessler's reagent and ring technique, which proved capable of detecting mercury in a dilution of 1-20,000. The haemocoele fluid of larvae which had been sterilized externally in this way was frequently tested for the presence of mercury, but none was ever found.

Preservation of material.

In the case of haemocoele fluid most frequently no preservative was necessary, as the material was generally used fresh. In our preliminary experiments with enzymes, the tissue was ground with fine sand specially prepared by treatment with nitric and hydrochloric acids, and subsequent prolonged washing and then drying. The tissue having been ground in this prepared sand, the resultant mass was dried in a dessicator. When required for use a portion of this mass was re-ground with normal saline solution and centrifuged ; the supernatant fluid was pipetted off and used for the test. This method of preserving tissue was abandoned, as some of the enzymes preserved in this way quickly lost their potency. In our later work the larva was dissected in normal saline and the required organs were transferred to a minute amount of 50 per cent. glycerine, in which they were kept until a sufficient number had been collected. They were then ground in a Hayden's bacterial mill and the resulting emulsion was stored on ice. From this concentrated suspension a small quantity was removed and diluted as required with normal saline solution. In all cases the final concentration of glycerine in the diluted fluid was very small. This method of preservation was found satisfactory for enzyme work, as no inhibition of enzymes attributable to the glycerine was noted in such dilutions.

III. PHYSIOLOGICAL INVESTIGATION OF LARVAL TISSUES AND FLUIDS

For the identification and estimation of enzymes we chiefly followed the techniques of Swingle (1925) and Wigglesworth (1927 and 1928).

I. SALIVARY GLANDS.

The reaction of the salivary glands was found to be pH 7·3 to 8·0.

Enzymes of the salivary glands. The only enzymes found by us in any notable amount in the salivary glands of Cordylobia larvae were (1) Amylase, and (2) Invertase.

(1) *Amylase.*

Technique of investigation. The glycerinated glands were ground to a fine emulsion which then diluted with normal saline. The method of demonstrating the presence of this enzyme consisted of incubating at 37° C. a mixture comprising :

(1) Ground salivary gland emulsion of third instar larvae, 0·5 c.c., 5 units (the glands of 1 larva = 1 unit).

(2) Substrate (2 per cent. starch solution), 0·5 c.c.

(3) Buffer solution, 0·5 c.c. (pH 6·8).

A control inactivated by heat was put up in each case. The incubating mixture was sampled at intervals by removing 0·2 c.c. and testing with iodine solution for the presence of starch and with Benedict's solution for the presence of reducing sugars.

Results. In all experiments with the salivary glands the presence of amylase was readily demonstrated. Using the quantities given above the end point of the amylolytic reaction as judged by the starch iodine test was reached as a rule within twenty-four hours.

Effects of guinea-pig serum on amylase.

Owing to the difficulty of obtaining salivary gland amylase free from admixtures and also to enable us to use large quantities for injection in the subsequent attempts at the production of anti-enzymes the tests of the effect of guinea-pig serum on amylase were carried out with commercial amylase (Martindale).

The serum of guinea-pigs did not exert any inhibitory effect on commercial amylase. On the contrary we found that normal guinea-pig's serum augmented the velocity of the amylolytic reaction. Further investigation showed that there exists in guinea-pig serum

an appreciable amount of amylase. The results obtained by incubating amylase, amylase and heated serum, and amylase and unheated serum with the starch substrate are shown in Graph I.
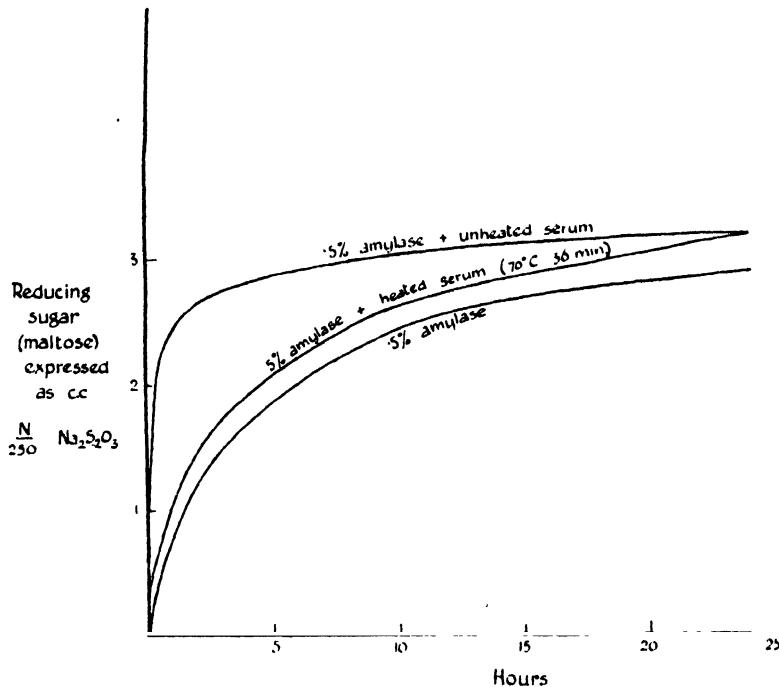
GRAPH I. SHOWING THE PRESENCE OF AMYLASE IN GUINEA-PIG SERUM.

Attempts to produce antiamylase in guinea-pigs.

Attempts to produce antiamylase in guinea-pigs by the injection of commercial amylase did not prove successful. The injections were made intraperitoneally in amounts varying from 0·1 c.c. to 4·0 c.c., the largest total amount injected being 10 c.c. in four injections. No ill effects from the injections were noted.

(2) *Invertase.*

Technique.

The following mixture was incubated at 37° C.

Ground salivary gland emulsion of third instar larvae, 0·5 c.c., 5 units (the glands of 1 larva = 1 unit).

Substrate (2 per cent. sucrose), 0·5 c.c.

Buffer solution, 0·5 c.c.(pH 5).

As before, 0·2 c.c. amounts were withdrawn from the incubating mixture at stated intervals and tested for the presence of reducing sugars by Benedict's method.

Results.

The occurrence in the salivary glands of Cordylobia of a powerful invertase was demonstrated in all the experiments

Effects of guinea-pig serum on invertase.

Commercial invertase was used ; no evidence was obtained of the presence of invertase in the serum of guinea-pigs; nor did the serum of guinea-pigs exert any inhibitory action on the invertase used. In a few experiments, however, it was noted that serum appeared to have a definite accelerating effect when dilute solutions of invertase were used.

Attempts to produce anti-invertase in the serum of guinea-pigs.

Injection of commercial invertase into the peritoneal cavity of two guinea-pigs did not result in the appearance of any anti-invertase. The dosage varied from 0·2 c.c. to 5 c.c. ; the total amounts injected were 6·2 c.c. in two injections and 6 c.c. in seven injections.

Repeated tests for maltase, lactase, pepsin and tyrosinase were negative ; trypsin and erepsin were found in mere traces after thirty-six hours' incubation. Thus amylase and invertase were the only two enzymes found in considerable amount in the salivary glands ; no inhibitory effect on these enzymes by either normal or immune guinea-pig serum was observed ; further, no antienzymes could be produced by injection of the corresponding commercial enzymes.

In our previous paper we referred to the possible utilization by the first instar larva of the salivary secretion as an aid to penetrating the stratum corneum of the host. Sitovosky (1905) showed that the larva of the clothes moth was capable of digesting keratin, and it occurred to us that the salivary secretion of Cordylobia besides being a lubricant, might contain some enzyme endowed with similar powers. Experiments were carried out with salivary glands, using as a substrate, skin peelings from a case of sunburn. The incubated mixtures showed no rise in acidity with Sorensen's method.

Other properties of the salivary glands.

Toxicity. It was observed that subcutaneous injection of salivary glands into guinea-pigs resulted in a very severe local and general reaction. The results of injection of salivary glands are shown in Table I below.

TABLE I.

Showing results of subcutaneous injection into guinea-pigs of the salivary glands of the third instar larva of *Cordylobia antbropophaga.*

Number of animals	Number of injection and number of larvae of which the glands were injected						Result
	1	2	3	4	5	6	
1	2	3½	5	6½	4	6	Lived.
2	2	...	...	...	...	...	Died, 7 days.
3	2½	4	4½	...	...	...	Lived.
4	3	3½	7	4	6	...	Lived.
5	4	...	...	...	...	...	Died, 24 hours.
6	6	...	...	...	...	...	Died, 3 days.
7	6	...	...	...	...	...	Died, 24 hours.
*8	6	...	...	...	...	...	Lived.

* Glands boiled for ten minutes before being injected.

Controls of equal bulk of normal saline and 50 per cent. glycerine were found to be non-toxic, although a slight transient local reaction to 50 per cent. glycerine was noted. Even one gland produced a definite local reaction with swelling and oedema, while on two occasions injection of the glands of four and six larvae respectively resulted in the death of the animals within twenty-four hours. In no case was it possible to inject as a first dose the untreated glands of four or more larvae into a guinea-pig without causing death. On the other hand, by proceeding with graduated doses, complete immunity to the toxin was readily obtained. In such animals the subsequent injection of the glands of six third instar larvae usually produced no reaction whatsoever, local or general.

The toxin in the salivary glands is destroyed by boiling for ten minutes, as shown in the case of animal No. 8 in the table ; here

the glands of six larvae were injected as a first dose without marked reaction.

The observations of Schwartz (1923) on the toxin of *Ascaris vitulorum* showed that saline extracts of this nematode contained a toxin which was thermostable to the extent that it could be brought to boiling point without losing its toxicity.

No haemolysin, haemagglutinin or coagulin was found ; an anti-coagulin which delays clotting for considerable periods is still under investigation.

Precipitin. The serum of normal guinea-pigs, immune guinea-pigs and of guinea-pigs previously vaccinated with large doses of salivary gland emulsion gave no precipitin reaction when subsequently tested with salivary gland antigen.

Eosinophilia.

Salivary glands were injected subcutaneously into guinea-pigs in varying amounts. In some cases a single injection, in others a total of two or three injections and in others a long series of injections were given. Among the last group of guinea-pigs, one received the glands of thirty-seven, and another those of forty-two third instar larvae ; in no case was any increase of the eosinophil percentage observed.

Immunizing properties of salivary glands.

The guinea-pigs referred to under eosinophilia were all tested for immunity by the application of living larvae. In no case was definite immunity established by the injection of salivary glands.

II. THE GUT.

For the purpose of these investigations the gut was considered in three portions : (1) Foregut, extending from the oral aperture to just above the enteric caeca ; (2) The midgut, from above the caeca to immediately above the insertion of the malpighian tubes ; (3) The hindgut, including the malpighian tubes to the anus.

(1) *Foregut.*

Enzymes.

Technique and results. As it was not feasible to weigh the different tissues dissected we cannot express our results quanti-

tatively. The findings as regards the relative concentration of the enzymes are not strictly comparable, because the incubating tubes did not contain equal weights of the glands, foregut, midgut, or other organ under observation ; the tubes did however contain equal numbers of these organs.

Both amylase and invertase were found in the foregut, but in very weak concentration. The effects on amylase and invertase of guinea-pig serum have already been mentioned under salivary glands, as have also the negative results of efforts to produce antienzymes and the lack of immunizing power. No maltase, lactase, pepsin, trypsin or tyrosinase was discovered in the foregut. Erepsin in faint traces was found.

Other properties of the foregut.

No haemolysin, coagulin, anti-coagulin or agglutinin was found in the foregut. In the study of precipitins, eosinophilia, toxicity and immunizing properties, the foregut was not treated separately from the rest of the intestinal tract.

(2) *Midgut.*

Reaction. The pH of the midgut was found to vary between 6·7 and 7·8.

Enzymes. The presence of amylase, invertase, maltase, trypsin, erepsin and lipase was demonstrated. Pepsin and tyrosinase were not found.

Technique. The methods used for detecting the presence of amylase and invertase have already been described.

Maltase. This ferment was tested for by digestion of maltose, the product being identified by the glucosazone crystal test and Barfoed's reagent.

Trypsin. Early experiments were unsatisfactory because the mixtures were not constantly shaken at the required temperature. The tubes were later incubated in a shaking apparatus kept at a constant temperature of 37° C. This apparatus, described to us by Dr. V. B. Wigglesworth, consisted of a tube carrier made of wire gauze fixed on a pivot in an agglutination bath. This carrier was provided with a vertical metal arm connected with a see-saw balance actuated by a water drip. The shaking of the mixture was found to increase the degree and velocity of the enzyme reaction. The

first method of estimating tryptic activity which we employed was the fibrin congo red method of Roaf ; this was found to give inconstant results, the heated control frequently parting with the dye before the experiment was terminated ; we therefore gave up this method. The casein method of Fuld and Gross, after preliminary experiments, was also abandoned in favour of Sorensen's method, by which the formol acidity was determined.

The estimations were made as follows :—Solutions required : (1) Formalin, 50 per cent., i.e., ordinary 40 per cent. commercial formaldehyde plus an equal volume of water, rendered neutral with NaOH ; (2) Phosphate Buffer solution at pH 8·3 ; (3) Standard colour indicator. For this, Clarke and Lubs Borate Buffer pH 9·0 was used (5 c.c. Borate Buffer and 5 drops, delivered from a standard pipette, of 0·05 per cent. Phenolphthalein in 50 per cent. alcohol). This standard indicator was kept in the dark and made up fresh every three or four days ; (4) Gelatin, 10 per cent., stored sterilely in small ampoules.

Two tubes, one containing heated trypsin and the other unheated trypsin were put up as follows :—

Solution	Tube I	Tube II
Tissue emulsion (0·1 c.c., 1 unit)	0·5 c.c. Unheated	0·5 c.c. Heated in boiling bath, 1 hour
10 per cent. Gelatin	0·5 c.c.	0·5 c.c.
Phosphate Buffer, pH 8·3	0·7 c.c.	0·7 c.c.

The tubes were shaken during the incubation period as described. At suitable intervals, 0·1 c.c. was removed from each of the tubes and added to 5 c.c. distilled water in a comparator tube, five drops of 0·05 per cent. Phenolphthalein in 50 per cent. alcohol were added and the solution adjusted to the colour of the standard by the addition of alkali ; 0·5 c.c. of formalin solution was added and the mixture titrated with $\dfrac{N}{100}$ NaOH delivered from a micro-burette. The formol acidity figure is obtained by subtracting the amount of $\dfrac{N}{100}$ alkali required by the tube containing the heated trypsin, from that required by the tube containing the unheated trypsin.

Erepsin was also tested for by the formol acidity method, the substrate used being 2 per cent. Wittes peptone in place of the gelatin used for trypsin estimations.

Solution	Tube I	Tube II
Tissue emulsion	0·2 c.c. Unheated	0·2 c.c. Heated in boiling bath, 1 hour
Witte Peptone, 2 per cent.	0·5 c.c.	0·5 c.c.
Buffer pH 8·3	0·4 c.c.	0·4 c.c.

Wigglesworth (1928) points out that in order to avoid an overlapping with the trypsin action, it is best to use as a substrate for erepsin (peptidase) the dipeptide glycyl-tryptophan, which is not acted upon by trypsin.

Lipase. The use as a substrate of neutralised olive oil, whether the mixtures were kept in a shaker or not, gave contradictory results. Later, ethyl butyrate was used as a substrate and gave constant results. Strictly speaking, the enzyme thus estimated was not lipase but butyrase.

Solution	Tube I	Tube II
Tissue emulsion	1·0 c.c. Unheated	1·0 c.c. Heated in boiling bath, 1 hour
Ethyl butyrate neutralized	0·2 c.c.	0·2 c.c.
Buffer pH 6	0·3 c.c.	0·3 c.c.

The tubes were incubated at 37° C., in the shaking machine, 0·2 c.c. amounts being removed at intervals and tested for free acidity. The difference between the amount of alkali required for the two tubes to bring them to the standard was taken as the acidity produced by the lipase.

Results.

It may be stated here that the enzymes amylase, invertase, maltase, trypsin, erepsin and lipase were present in their most concentrated form in the midgut.

Effects of guinea-pig serum on the enzymes of the midgut.

The effects of guinea-pig serum on amylase and invertase have already been considered ; no experiments were done with maltase, or erepsin.

Trypsin. Normal guinea-pig serum incubated, with toluol added as preservative, showed no tryptic auto-digestion as estimated by a rise in the formol acidity. This might be due either to absence of trypsin or to trypsin being balanced by antitrypsin present in the serum. The presence of antitrypsin was readily demonstrated in both normal and immune guinea-pig serum, the trypsin used for its demonstration being Martindale's trypsin 10 per cent. In graph No. II are shown the curves representing digestion of heated and
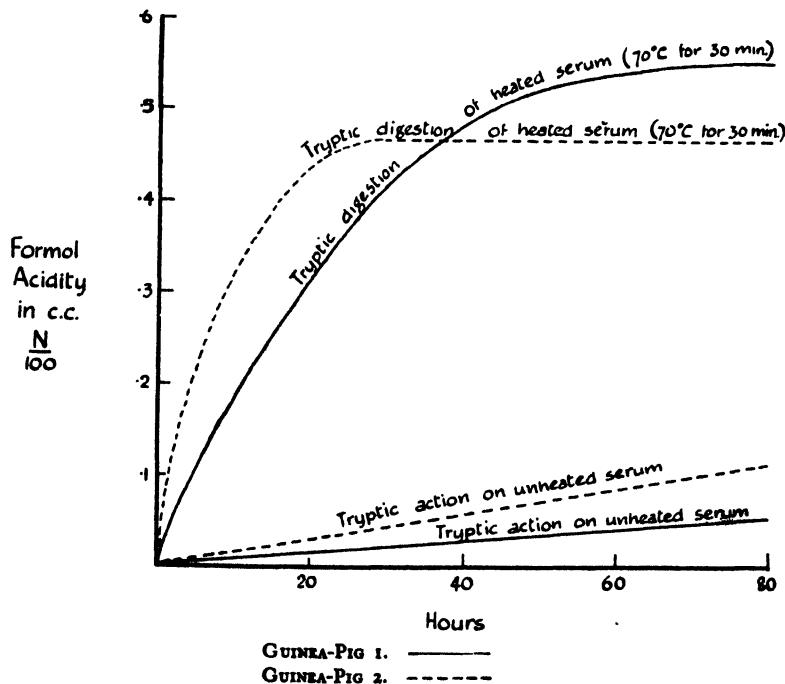
GRAPH II. SHOWING PRESENCE OF HEAT LABILE ANTI-TRYPTIC SUBSTANCE IN GUINEA-PIG SERUM.

unheated sera by 10 per cent. Martindale's trypsin. It is seen that the curve representing the digestion of heated serum has a much higher level than that representing the digestion of unheated serum.

Lipase. Guinea-pig serum proved highly lipolytic. Thus, as shown in Graph No. III, the serum of a guinea-pig in 10 per cent.
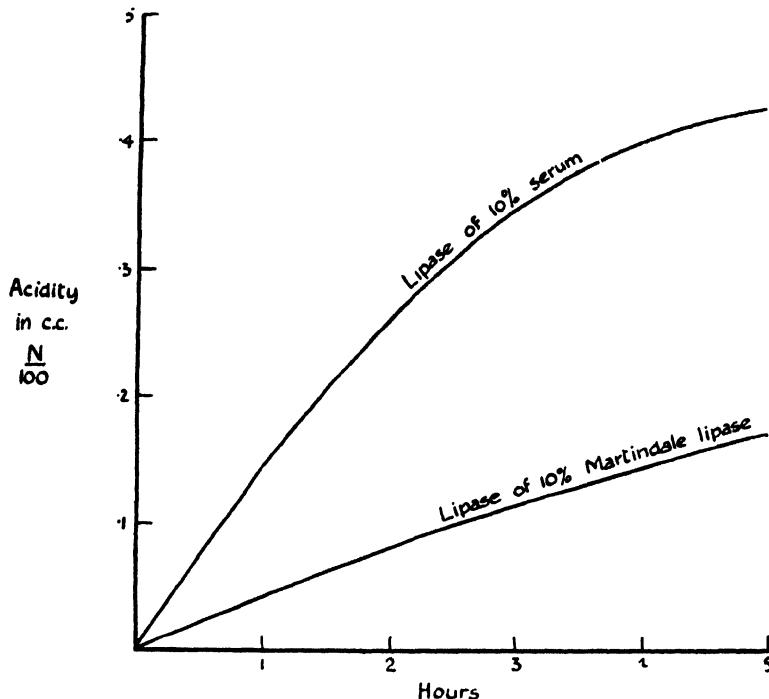
GRAPH III. SHOWING DIGESTION OF ETHYL BUTYRATE BY NORMAL GUINEA-PIG SERUM.

dilution was approximately twice as active as the 10 per cent. solution of Martindale's lipase which was used as a control. Similar curves were obtained with the sera of other guinea-pigs.

Efforts to produce anti-trypsin and anti-lipase in guinea-pigs by injection.

Neutralized liquor pancreaticus Benger was used for injection, the first non-neutralized injections having proved rapidly fatal. The trypsin solution wàs injected into four guinea-pigs by the intra-peritoneal route ; the largest total amount received by one animal was 7 c.c. of Martindale's trypsin in four injections extending over a period of a month.

The first effect of injection of large amounts of trypsin was a rapid lowering of the anti-tryptic index of the serum, the fall being

succeeded in about a day by a rise, which was of a definite but temporary nature with considerable fluctuations. In three guinea-pigs which received various amounts of trypsin in a series of intra-peritoneal injections, the anti-tryptic index (i.e., the rise in acidity in the heated tube divided by the rise in acidity in the unheated tube) rose in the first case from 0·33 to 0·93, in the second case from 0·51 to 1·0, and in the third case from 0·67 to 1·0. It is clear from the above figures that intraperitoneal injection of commercial trypsin in adequate amounts produces a rise in the anti-tryptic index of guinea-pig serum. This is so far the only series of experiments in which we have found that the injection of an enzyme produced anti-enzyme in the guinea-pig.

Anti-lipase. Of three animals injected with Martindale's lipase, only one survived sufficiently long for observations to be made. In the one animal which survived and which received 1·1 c.c. of Martindale's lipase at a single injection, the lipolytic power of the serum was not altered.

Other properties of the midgut.

Toxicity. No injections of midgut alone were made. but in several instances the midgut was injected with the body contents ; reference to the results of these experiments will be made later.

Haemolysins, agglutinins, coagulins and *anti-coagulins* were not found.

Precipitins. The midgut was not tested separately for precipitins.

Eosinophila.

The midgut was not injected separately in the endeavour to produce eosinophilia.

Immunising properties of the midgut.

The midgut was not injected separately in the endeavour to produce immunity.

(3) *Hindgut.*

The pH of the hindgut was found to lie between 7·7 and 8·0. The following enzymes were found in weak concentration, amylase, invertase, lipase and erepsin. No haemolysins, agglutinins or

coagulins could be demonstrated. An anti-coagulin was found to be present and is still undergoing investigation.

In our detailed considerations of the fore, mid and hindguts we have omitted experimental results regarding toxicity, precipitins, eosinophilia and immunising properties, because these experiments were carried out with the whole gut and not with individual portions of it. The results of the experiments were as follows :—

Toxicity of the gut. Injections of the unheated gut proved highly toxic. The gut of even one third instar larva produced a very severe local and general reaction which in one case proved fatal within forty-eight hours. In experiments carried out with similar but preserved material which had been stored in 50 per cent. glycerine, or else filtered through a Seitz filter, different results were obtained. Very slight local reaction followed the injection of large initial doses of such material and subsequently the animals tolerated still larger doses. For instance one guinea-pig received in five injections the intestinal tracts of twenty-three larvae without ill effects.

Precipitins. On only one occasion was a weak positive reaction obtained, using the hindgut as the test antigen. The animal showing the precipitin had acquired immunity as a result of a long series of experimental infections. The larval coelomic fluid and faeces gave a much more marked reaction with the same serum. The serum of ône animal which had survived the reaction following the injection of the intestinal tract of a single third instar larva subsequently gave no precipitin reaction when tested against this antigen.

Eosinophilia. No eosinophilia was shown to result from the intra-cutaneous injections of larval gut, one to four untreated intestinal tracts being used. The observation period was only three days in two of the animals, but in the third, daily examinations were made for twenty-three days following the injections.

Immunizing properties of the gut. We have been unable to reach any conclusion regarding the immunizing properties of the gut, as the only two animals which survived the period of a month between the last of the injections and the test application of larvae, died during the six-day period of the test.

III. THE CUTICLE.

Intracutaneous injections of emulsions of larval skin produced very little reaction whether the cuticles were preserved in glycerine or simply ground fresh in normal saline. In this connection it is of interest to note that the single injection of 1,000 entire first instar larvae produced no reaction.

Only three animals survived the series of injections and subsequent period before testing. The dose given and the results of the testing by the application of larvae are detailed below.

GUINEA-PIG 119. The animal had a total of 8·5 cuticles of third instar larvae, intradermally in five injections over a period of one month. After three weeks a test with six larvae resulted in three developing to the sixth day.

GUINEA-PIG 125. Did not become immune after a total amount of twenty-seven cuticles of third instar larvae administered intradermally in seven injections over a period of six weeks. After an interval of three weeks it was tested with six larvae, of which two survived to the sixth day.

GUINEA-PIG 129. This animal had a total amount of forty-three cuticles of the third instar larva intradermally in six injections over a period of five weeks. After an interval of three weeks it was tested with six larvae, of which none survived to the sixth day. It seems that some degree of immunity can be produced in guinea-pigs by the intradermal injection of larval cuticle if very large doses are employed.

No precipitin reaction was observed between the sera of immune guinea-pigs and ground cuticle emulsions.

No eosinophilia was observed during a period of eleven days following the injection of the cuticle of one large third instar larva. The blood examinations were carried out daily.

IV. THE HAEMOCOELE FLUID.

The average amount of haemocoele fluid obtained by puncture of the third instar larva was 0·025 c.c.

Reaction. The pH of the haemocoele fluid varied from 6·7 to 7·5.

Enzymes. The only enzyme so far tested for in this fluid is tyrosinase ; it was found present in all instars When haemocoele

fluid of third instar larvae was released from the body it soon turned black if allowed to remain in contact with air. This blackening is due to the presence of tyrosinase and tyrosin, the inter-action of which results in the productions of a black pigment presumably melanin.

Results. If free-living first instar larvae taken direct from sand are ground up with a drop of 0·1 per cent. tyrosin, slight blackening occurs, indicating the presence of tyrosinase. No spontaneous blackening in the absence of added tyrosin was ever noted ; the first instar larva therefore contains tyrosinase but no tyrosin. When larvae were applied to an animal and then extracted after various periods the amount of larval tyrosinase was found to have markedly increased, but tyrosin could not be found up to the time of, or during, the first ecdysis. In spite of this apparent absence of tyrosin, the spines of the second instar soon become black or very darkly pigmented, and if it is the case that such blackening is due to melanin arising from the inter-action of tyrosinase and tyrosin, we must conclude either : (1) That the tyrosin required for purposes of pigmentation is so small in amount that we cannot detect it by the tyrosinase test, or (2) That the tyrosin is plentiful but is present in such a form that it does not blacken with tyrosinase.

As we shall show when dealing with the third instar, tyrosin can be so altered by contact with haemocoele fluid that it is no longer capable of reacting with tyrosinase to produce melanin.

Tyrosinase is very plentiful in the haemocoele fluid of the second instar and here again as in the first instar, it is unaccompanied by any tyrosin detectable by spontaneous blackening on exposure to air. Third instar larvae usually possess large quantities of both tyrosin and tyrosinase, but one third instar larva, immediately after its ecdysis from the second instar, was found to contain no tyrosin when the ecdysis took place *in vitro*.

Distribution of tyrosinase.

The property of blackening possessed by insect larval tissues is well recognised, and there are frequent references to it in the literature. That this blackening is due to the action of the haemocoele fluid only, as distinct from the general tissues, has not, however, hitherto been recognised. In the case of Cordylobia

larvae, the absence of tyrosinase from tissues other than the haemocoele fluid was shown by us in the following manner. Five third instar larvae were dissected under a constant stream of normal saline solution, care being taken not to puncture the gut until the washing was complete, and then the salivary glands, foregut, midgut, fatbody, and hindgut were dissected out, ground up and made up to 0·5 c.c. amounts with saline in incubating tubes. To each of these tubes were then added 0·5 c.c. of 0·1 per cent. tyrosin and 0·6 c.c. of Buffer pH 7·1. Two additional tubes, one containing haemocoele fluid and the other potato tyrosinase, instead of the tissues, were included in the series as positive controls. The negative controls were similar tissue emulsions set up in a similar manner but heated to 100° C. for one hour. It was previously found that the presence of even minute traces of tyrosinase could be detected in the incubating mixture containing tyrosin, if air were continuously bubbled through it. In the above experiment, therefore, the tubes were placed in the incubator and each was connected up with an air bubbling apparatus; the apparatus was kept running for 18 hours. The contents of the tubes were examined at intervals and it was found that the unheated haemocoele fluid and potato tyrosinase tubes blackened rapidly; but in none of the other tubes was any blackening discernible even after eighteen hours.

Distribution of Tyrosin.

We have shown that tyrosin is present in large quantity in the haemocoele fluid of the third instar larva. As it is almost completely absent in the pupa, it is presumably used up in the pigmentation of the pupa case.

Individual portions of the larva were tested for tyrosin, the necessary dissections being made, as before, in a stream of normal saline. After this dissection the entire intestinal tract and salivary glands with fatbody and portions of the tracheal system were immersed in pupal emulsion made with distilled water in order to facilitate penetration. The pupal emulsion had previously proved very rich in tyrosinase and devoid of tyrosin. No blackening whatsoever was observed to take place in the intestinal tract or salivary glands; marked blackening was observed in the tracheal system and also, at first inspection, parts of the fatbody appeared

to be blackened. On further examination it was found that the blackening of the fatbody was, in fact, closely related to the tracheal system and was due to pigmented particles within and to a less degree outside the lumen of the tubes, especially at their junction with the fatbody (fig. 2). This phenomenon was apparently due to
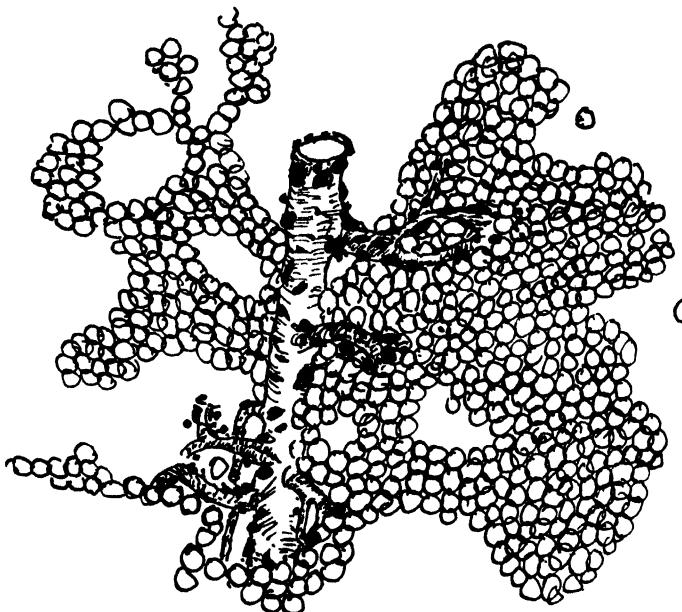
Fig. 2. Showing melanin around tracheal tubes in fatbody.

portions of the severed tracheal branches having become filled with the haemocoele fluid during the dissection, so that even the continuous and prolonged washing described was unable to remove it from the tubes.

Our experiments would appear to show that the tyrosin and tyrosinase in the third instar larva of Cordylobia are confined to the haemocoele fluid. But .these findings are based on the absence of melanin production and we have considerable experimental evidence to show that a fluid which contains tyrosinase may act on tyrosin in such a way that it will no longer blacken either spontaneously or on the addition of more tyrosinase. Thus haemocoele fluid alone, or a mixture of pupal emulsion and tyrosin which normally blacken readily on exposure to air, did not so blacken if deprived of oxygen

either by sealing it in a tube anaerobically, or exposing it to an atmosphere devoid of oxygen. We anticipated that on subsequently exposing this fluid to air, it would at once blacken. This, however, was not the case. That this failure to form melanin was not due to a deterioration in the enzyme tyrosinase, was proved by the fact that on the addition of fresh tyrosin solution, the fluid quickly blackened in a normal manner. It appeared certain, therefore, that it was the tyrosin which had undergone change. The shortness of the incubation period—three hours being sufficient to deviate the tyrosin—appeared to exclude the action of bacteria as a cause of this phenomenon. That bacteria can alter tyrosin is well known, and experiments which we carried out with certain strains of B. *pyocyaneus*, encountered in the same guinea-pig's serum on three occasions, showed that this organism had the power of rendering tyrosin undetectable by the tyrosinase test ; but the bacterial action was slow because even when we mixed concentrated living cultures of the organism with tyrosin at 37° C., blackening on the addition of tyrosinase was still detectable in one of the experiments for as long as seventeen hours. This tyrosin altering power was also found to exist in guinea-pig serum whether normal or immune.

Sources of the larval tyrosin.

As we have seen, the first, second and third instars all contain tyrosinase, whereas only the third contains tyrosin in a form detectable by the tyrosinase reaction. Yet it is almost certain that even the first and second instar larvae must contain some tyrosin ; as has already been mentioned, the blackening of the spines after the first ecdysis presumably indicates its presence ; yet larvae extracted from an animal immediately prior to this did not possess any demonstrable tyrosin. Now we have demonstrated the fact that the skin of guinea-pigs contains tyrosin, by snipping off small portions of the skin, and emulsifying them in a solution of tyrosinase from Cordylobia pupae. Tests were performed, using (1) skin of guinea-pig plus water, (2) skin of guinea-pig plus pupal tyrosinase, (3) pupal tyrosinase alone. Definite blackening occurred in the emulsion of skin in the pupal tyrosinase, whereas no blackening occurred in the controls. Tyrosin was found in immune as well as in normal guinea-pig skins. Therefore, larvae from the moment of

their first penetration of the skin are in contact with tyrosin, which forms a large percentage of the keratin of the corneal layers of the skin.

It seems certain, therefore, that larvae immediately after penetration of the skin of the host, take up tyrosin from the skin and they probably also store it, but in such an altered form that it does not blacken when tyrosinase is applied to it.

Effects of guinea-pig serum on tyrosinase.

Both normal and immune sera were found to diminish the activity of the tyrosinase, but this inhibitory power was not greater in the immune than in the normal guinea-pig serum. A suspension of tyrosinase made from pupal emulsion was incubated with normal and immune guinea-pig serum. After varying periods of incubation at 37° C., samples of each mixture were tested for the persistence of the enzyme. It was found that the tyrosinase in the presence of serum, whether normal or immune, diminished in activity as compared with a control tube containing normal saline and tyrosinase in place of serum and tyrosinase.

Efforts to produce anti-tyrosinase in guinea-pigs.

Two guinea-pigs were injected intracutaneously with haemocoele fluid of third instar larvae. The first, a local guinea-pig, received the haemocoele fluid of five larvae in two injections during fourteen days. The second, an English non-immune guinea-pig, received the haemocoele fluid of four larvae in two injections in ten days. Both sera inhibited the action of pupal tyrosinase, as compared with the saline control, but no difference in the degree of inhibition was noted between these sera and the serum of a control English guinea-pig.

Other properties of the haemocoele fluid.

Toxicity. The injection of haemocoele fluid intradermally resulted in a marked local and general reaction, proportional to the dose injected. The haemocoele fluid of one third instar larva resulted only in a slight local reaction, but the injection of four times this dose was followed by marked local reaction and death within a few days. As in the case of salivary gland injections, immunity to the toxin was rapidly acquired ; an animal, after receiving a series

of graduated doses, tolerated the injection of the haemocoele fluid of five full-grown third instar larvae with only a subsequent slight local reaction. This reaction is in all probability not due to the presence of bacteria in the haemocoele fluid, as we have found that this fluid is relatively free from bacteria ; thus, out of eight larvae dissected with sterile precautions, the haemocoele fluid proved to be entirely sterile in three cases.

Haemolysins, coagulins and *anti-coagulins* were tested for with negative results ; a weak agglutinin was demonstrated.

Precipitins. In a previous paper, Blacklock and Gordon (1926), we recorded the fact that we had been unable to demonstrate precipitins to Cordylobia antigen in the serum of immune guinea-pigs. We stated, ' Numerous experiments with immunized guinea-pig serum and ground-up larvae of all ages were completely negative ' ; we also recorded that similar experiments with partially immunized rabbits were unsuccessful. Since the publication of the paper referred to we have frequently repeated these experiments, chiefly with first instar larvae and always with the same negative results. We have now proceeded further and tested for precipitin, using various portions and organs of the larva, especially of the third instar. The results as regards the tissues were negative, with the exception of a very feeble precipitin obtained occasionally with gut and fatbody, when tested with the serum of guinea-pigs which had previously had many applications of larvae. Tests carried out with salivary glands and cuticle emulsion were negative when the serum of guinea-pigs immunised by the usual series of larval applications was used. When the haemocoele fluid itself was examined, it was discovered that it gave a strong precipitin reaction with the serum of imported guinea-pigs which had previously had larvae applied to them and also with that of many of the local guinea-pigs which had received no larvae experimentally, but of which the previous history was uncertain. We have already shown that local guinea-pigs enjoy a certain degree of immunity and we suggested that this was probably due to a previous natural infection.

It will be recalled that the dissection technique used by us involves free washing of the haemocoele cavity in order to get rid of the blackening effect of the haemocoele fluid on the tissues of the larva. As a result of this, until we came to the point of testing the

haemocoele fluid itself for precipitating power, this portion of the larva had carefully been excluded in our tests of tissues.

It appears that certain portions of the larvae are by no means strongly antigenic because when we injected salivary glands even in considerable amounts, the animal's serum did not give any precipitin with the specific antigen employed. The same negative results were obtained with gut and fatbody, but in these cases, on account of the extreme toxicity of the injection, small amounts only could be used.

In contradistinction to these negative results, a relatively small injection of haemocoele fluid causes the serum of the animal to give a very definite precipitin reaction with haemocoele fluid. In view of the importance of this discovery we have devoted considerable time to its investigation and we propose to discuss the results of our experiments in some detail.

Technique and results of precipitin reaction with haemocoele fluid.

The haemocoele fluid was removed from third instar larvae anaesthetised with chloroform ; a measured volume was taken and diluted with normal saline solution to 1-100, which was found to be the optimum dilution. In a series of tests using known positive sera, the precipitin test was markedly positive even when the haemocoele fluid was diluted 1-800. The haemocoele fluid diluted 1-100 was found to retain its precipitating power when kept in the ice chest up to a month; it is probable that it will retain its potency for considerably longer periods.

The tyrosinase-tyrosin reaction begins to take place in the fluid soon after its withdrawal from the larva, and even the 1-100 dilution shows a definite blue-black colouration ; the melanin formation, however, does not interfere with the precipitin reaction, which occurs independently of it ; this was shown to be the case by experiments carried out with pupal emulsions which give a precipitin reaction, but which contain no appreciable amount of tyrosin and, therefore, do not blacken. As regards the serum, it was found that the reaction was most marked when undiluted serum was used, and that although the test was still positive up to a serum dilution of 1-8 in normal saline, it grew progressively weaker with dilution. The ring-test was employed, haemocoele fluid in the selected dilution being carefully layered with a fine pipette on to the surface of the

undiluted serum in small tubes; the results were read after five minutes at room temperature and again after fifteen minutes at 37° C.

Results.

We examined a series of imported English guinea-pigs immediately after their arrival in this country; none of these gave a positive precipitin reaction with haemocoele fluid   Six imported English guinea-pigs which were examined for precipitin after the development of larvae on them, all gave a positive reaction.   We then investigated the precipitin in the serum of local guinea-pigs which had no larvae placed upon them experimentally.   Twenty-three local guinea-pigs purchased at different places were tested for precipitin reaction. Of these guinea-pigs, fifteen gave a positive and eight gave a negative reaction, that is to say, that well over half of them would appear to have been previously infected by Cordylobia.   As it happened, there was in this batch of local guinea-pigs a larger proportion than usual of young animals under 250 grammes, otherwise the number of positives would almost certainly have been even greater than it was.   As in the case of English imported guinea-pigs, all the negative local animals gave a positive precipitin reaction when larvae were placed on them subsequently.   These facts are illustrated in Table II.

TABLE II.

Showing percentage of positive precipitin reactions with guinea-pig serum and haemocoele fluid (diluted 1-100) of *C. anthropophaga* third instar larvae.

Type of animal	Previous treatment	Number of animals tested	Percentage positive
Imported	Nil	50	0
Local	Nil	23	65
Guinea-pigs with negative precipitin reaction...	Larvae applied and allowed to develop.	11	100

In addition to the above, the following imported English animals were tested, viz.: two animals which had received injections of respectively 2,100 and 1,100 first instar larvae; one animal which had received an injection of intestinal tract and fatbody. All three animals gave negative precipitin results. As was to be expected, animals which had received injections of haemocoele fluid but had had no larvae applied, gave a positive result with haemocoele

fluid. It is noteworthy that the animal which had received 1,100 first instar larvae and then gave negative precipitin reactions with haemocoele fluid for a month following the last injection, gave a positive reaction within twelve days of the application of twelve larvae, of which four developed.

The positive precipitin reaction given by local guinea-pigs not experimentally infected appears to point clearly to a previous natural infection. It is such previous infection that we have already postulated in our earlier paper as being the probable cause of their relative immunity.

Further proof of the association of the relative immunity of local guinea-pigs with previous natural infection is obtained by a considera-tion of the proportion of larvae surviving to the sixth day in local guinea-pigs giving no precipitin reaction, and in local guinea-pigs giving a precipitin reaction. For example, five local animals which gave a negative precipitin reaction were tested with sixty-one larvae ; of these, thirty-one developed. Five local animals which gave a positive precipitin reaction were similarly tested with sixty larvae ; of these, only sixteen developed.

Although we have thus shown that the serum of guinea-pigs which have had larvae develop on them gives a precipitin reaction subsequently with haemocoele fluid, it must be clearly understood that the precipitin reaction is not necessarily proof of the existence of complete immunity. A guinea-pig which gives a positive precipitin reaction with haemocoele fluid may yet permit a proportion of larvae to survive as shown above. The reaction persists for at least two months, often the end of infection ; longer periods have not yet been tested.

Eosinophilia.

It was found that the injection of haemocoele fluid of third instar larvae produced a definite rise in the eosinophilia percentage in certain instances. In our previous paper we stated that cutaneous injection of ground-up third instar larvae was always followed by a marked rise in the eosinophil percentage, but if the table illustrating this fact is examined, it will be seen that this statement was made of animals in which the eosinophil count before treatment began varied from 1·3 to 4·9 per cent. In our present experiments we

injected haemocoele fluid into two imported and one local guinea-pig; during the short observation period of three weeks, no pronounced increase was noted in the case of the two imported guinea-pigs, both of which had, previous to injection, an eosinophil percentage below one. In the case of the local guinea-pig, a definite eosinophil rise followed each injection as shown below.

	Day										
	1	3	4	6	8	15	17	19	21	23	26
Injection ...	+	...	...	...	...	+	...	...	...	...	...
Eosinophil percentage ...	2·9	10·7	12·7	5·3	3·1	2·1	4·9	4·4	4·2	6·1	5·1

Immunizing properties of haemocoele fluid.

One local guinea-pig received in two intracutaneous injections on the abdomen the haemocoele fluid of five third instar larvae. It was tested twenty-five days later by applying to the abdomen six larvae, of which one developed. Of six larvae applied at the same time on the back, none developed. This being a local animal, the only conclusion to be drawn is that the haemocoele fluid in the dosage used would not produce immunity in an imported animal. An imported English guinea-pig received in five intracutaneous injections on the abdomen the haemocoele fluid of twelve third instar larvae. It was tested a month after the last injection with six larvae on the abdomen and six on the back ; of these, seven developed.

The relation of the haemocoele fluid to respiratory function in Cordylobia.

From the time the larva in the first instar penetrates the cuticle of the animal host, until the time it leaves the host prior to pupation, the larva normally keeps the aperture in the host's skin open so that its posterior spiracles are not occluded. As the larva grows, the aperture in the skin is enlarged and the posterior end of the larva can be seen at intervals alternately protruding and retracting.

In the skin of the immune or partly immune host, the larvae appear to lose their power of keeping open the aperture in the skin ;

however, even when the aperture is apparently completely occluded by a tenacious scab, the larva may sometimes be found alive beneath it. It appeared probable that the complete or partial occlusion of the larval stigmata thus produced must interfere with the insects' normal respiration, which necessitates a large supply of atmospheric oxygen. We made some experiments in order to obtain some idea of the amount of oxygen required by the normal larva ; we also made some observations on the length of time larvae could survive when deprived of oxygen ; a few experiments of a similar nature were also made with the pupa and the fly.

A rough estimate of the amount of oxygen required by the larva *in vitro* was made by means of a simple apparatus which is figured below (fig. 3) ; the larva was placed in short tube, *A*, and this was
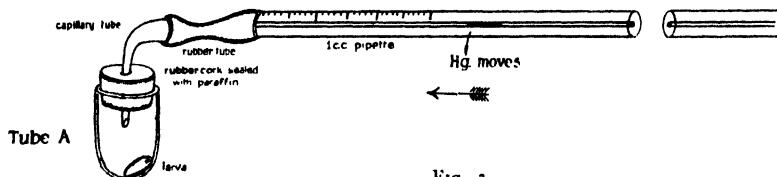
Fig. 3.

connected by fine glass and rubber tubing to the end of a 1 c.c. pipette graduated in 1/100ths. All joints were sealed with melted paraffin, and readings were commenced after the apparatus had had time to adjust itself to the room temperature.

In one experiment with the above apparatus it showed that in twenty-four hours a half-grown third instar larva used at room temperature, 0.45 of a c.c. of oxygen, but the actual amount of oxygen consumed was greater than this, as the apparatus did not provide for any estimation of carbon dioxide given off. We therefore modified the apparatus in such a way as to enable us to estimate the amount of carbon dioxide given off, as well as the amount of oxygen consumed. The diagram (fig. 4) below is self explanatory ; after the period of
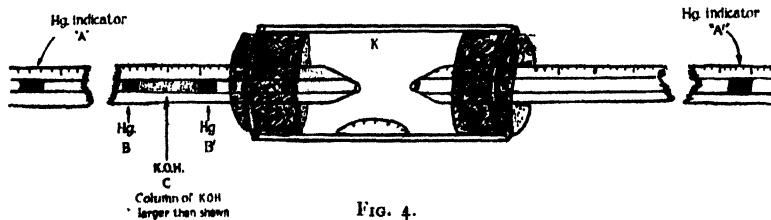
Fig. 4.

observation is completed, the movement of the mercury, A, and A^1, is read off ; then the two short columns of mercury, B and B^1, with the enclosed caustic potash solution, C, are delivered by suction into the tube, K, in which the larvae are enclosed. The carbon dioxide in the apparatus is rapidly absorbed and the second movement of the mercury in the graduated tubes can be read off. The second reading gives the amount of carbon dioxide present. The total oxygen consumed by the larva is the sum of the first and second readings. Using this apparatus we found that one large third instar larva in twenty-four hours took up 0·56 c.c. of oxygen and gave off 0·26 c.c. of carbon dioxide. The consumption of atmospheric oxygen by the pupa was found to average 0·15 c.c. in twenty-four hours. The absorption of oxygen by the adult fly when kept in a confined space and prevented from flying, was 0·06 c.c. in twenty-four hours.

When larvae were deprived of oxygen by being enclosed in an oxygen-free atmosphere, we found that they not only survived for long periods as shown below, but continued to give off carbon dioxide in appreciable quantities as long as they were alive. Thus, three third instar larvae gave off 0·15 c.c. carbon dioxide and two larvae gave off 0·2 c.c. in twenty-four hours, the amount per larva in twenty-four hours being 0·05 c.c. to 0·1 c.c. The apparatus used for the experiments is shown in fig. 5.
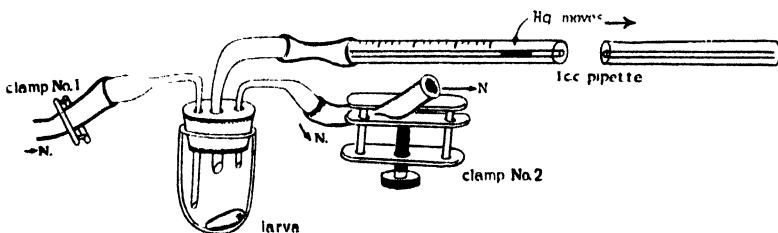
FIG. 5.

The length of life of the larva in an oxygen-free atmosphere.

Our first experiments were carried out by immersing larvae in liquid paraffin ; this method was soon given up as unsuitable. We found it better to make use of nitrogen gas by the following method. The larva to be tested was placed in a short length of glass tubing which was then connected with a nitrogen reservoir and the gas was allowed to pass through it. Sufficient gas was passed through the

tube to displace all the air and then by means of clamps on the rubber connections, the tube enclosing the larva in a nitrogen atmosphere was isolated, detached and placed in water so that any leak could be detected. We were surprised to find that larvae, especially of the third instar, were capable of survival in this oxygen-free atmosphere for long periods. In a series of experiments we found that third instar larvae gave more constant results as regards the survival period than did first instar larvae. In one of our experiments with six third instar larvae in an atmosphere of nitrogen, all the larvae survived for sixteen hours ; in another, they survived for nineteen hours, and in a third, a larva survived for twenty-seven hours.

A proportion of first instar larva survived a similar test for from one and a half to two and a half hours in three experiments. In an experiment carried on for nineteen hours, all the larvae, ten in number, found at the end of the experiment, were dead.

This remarkable power of surviving oxygen deprivation is much less evident in the fly than in the third instar larva ; flies survived six hours but failed to survive ten hours or longer. Again, while the larva continued to move in a fairly normal manner in the oxygen-free atmosphere for many hours, the fly became quiescent within a few seconds and remained motionless thereafter. On re-admitting oxygen, the flies which recovered did so slowly, the first movements being seen after about one hour, whereas larvae exposed for much longer periods, if they had become quiescent, recovered rapidly. We observed in one experiment a larva pupate in an oxygen-free atmosphere ; the experiment was as follows :—Two third instar larvae and one adult fly were placed in an oxygen-free atmos-phere at the same time, each in a separate receptacle. The fly was removed after the lapse of ten hours and was found to be dead. At the end of twenty-two hours one larva was still moving actively, but the other had begun to pupate. After forty-eight hours the pupa and the larva were removed, the former having darkened normally in the nitrogen atmosphere. The larva did not recover, but the pupa after this forty-eight hours' exposure to an oxygen-free atmosphere, was kept under observation ; from it, twelve days later, a normal fly emerged. On the other hand, a pupa which had not yet begun to blacken was placed in the same atmosphere for three

days, during which time no blackening occurred. On re-admitting air at the end of the three-day period, the puparium at once blackened normally. In the case of this pupa the fly did not subsequently emerge.

It seemed to us highly improbable that the usually described mode of respiration in insects which are not provided with an oxygen carrying circulatory system could suffice to account for such prolonged survival in an oxygen-free atmosphere. For on this accepted theory of respiration the only oxygen available for the tissues in the larva of Cordylobia would be the relatively minute quantity actually contained in the tracheal system of the larva at the time of exposure to the oxygen-free gas.

We have already shown that Cordylobia larvae in the third instar contain large amounts of both tyrosinase and tyrosin and we have proved by a series of experiments with each organ and tissue that these substances are confined to the haemocoele fluid, since they cannot be demonstrated in the tissues when they have been washed free of haemocoele fluid. The function usually ascribed to the enzyme tyrosinase and its substrate tyrosin in insects is that of providing, by a process of oxidation, melanin for the darkening of the spines of the different instars, the blackening of the pupa case, and the pigmentation of the imago.

The process of melanin formation by the interaction of tyrosinase and tyrosin in air results in a considerable absorption of oxygen, as shown by the experiments described below. Since third instar haemocoele fluid contains tyrosinase and tyrosin, it commences to blacken very soon after the liberation of fluid from the larva. In order to carry out the experiments with greater accuracy, we first made use of an emulsion of the pupa which contains tyrosinase but no tyrosin, and a solution of commercial tyrosin. The experiments were carried out in an apparatus similar to that used for estimating the oxygen absorbed by the living larva. When pupal emulsion was incubated with tyrosin it was found that oxygen was absorbed from air by the mixture in relatively large amount. The emulsion of half of a pupa in 0·5 c.c. of normal saline solution mixed with an equal volume of 1-1,000 tyrosin solution in distilled water took up and apparently stored in twenty hours, 0·4 c.c. of oxygen from the air ; on drawing the residual gas through lime water, a white

precipitate occurred, indicating the presence of carbon dioxide ; the amount of oxygen absorbed was, therefore, actually greater than that shown. The experiment was then repeated, using haemocoele fluid, and demonstrated the fact that the haemocoele fluid, by itself, is capable of respiring in a manner comparable to the living larva, i.e., it takes up oxygen and gives off carbon dioxide. It was found the haemocoele fluid of one larva, if rapidly transferred to the apparatus, absorbed from 0·06 to 0·1 c.c. of oxygen in twenty-four hours. The carbon dioxide output of the haemocoele fluid was estimated by the apparatus already illustrated in fig. 5. Using this apparatus, we found that the haemocoele fluid collected from six larvae produced approximately 0·05 c.c. of carbon dioxide in twenty-four hours. We ruled out the possible effects of bacterial action by controls consisting of pupal emulsion, i.e., tyrosinase, and a solution of tyrosin in separate tubes ; in neither of these cases was gas absorbed or given off. Again, pupal emulsion prepared with sterile precautions, mixed with boiled tyrosin in a sterile apparatus, gave identical results with experiments in which sterile precautions were not taken. The controls served the additional purpose of showing that neither pupal emulsion alone, nor a solution of tyrosin alone absorbed oxygen from the air.

It seemed important to us that although the tyrosinase-tyrosin mixture had taken up such a relatively large amount of oxygen, it showed blackening only to a slight degree, at the end of the experiment.

The fact that third instar haemocoele fluid is able to absorb from the air a quantity of oxygen and store it, suggested to us a possible association of this function of the haemocoele fluid with the proved power of the larva to maintain itself for a considerable time in adverse conditions when the supply of atmospheric oxygen is cut off. We believe that the larva of Cordylobia, during its normal respiration, deviates a certain proportion of the oxygen of the inspired air, by means of its haemocoele fluid, to form a compound containing oxygen in a form which can be utilised by the tissues when the normal supply is cut off. If such a mechanism of subsidiary or by-pass respiration exists in the haemocoele fluid of Cordylobia larvae, it would be reasonable to expect some change to occur in the amounts of tyrosinase or tyrosin in the haemocoele fluid, when the larva is deprived of oxygen for long periods.

Our experiments show that such a change does occur in the haemocoele fluid of larvae when they are exposed for any length of time to a nitrogen atmosphere. It was found in experiments that the haemocoele fluid of nitrogen-treated larvae invariably failed to blacken in the normal manner when exposed to the air ; at the most, the colour change was to a reddish brown hue quite distinct from the blue-black appearance of normal haemocoele fluid after exposure to air.

In a series of experiments we investigated the cause of this change in the haemocoele fluid after oxygen starvation. Third instar larvae were enclosed in a nitrogen atmosphere for various periods, and the haemocoele cavity then opened and the fluid tested. In larvae examined after from one and a half to seven hours in nitrogen, the haemocoele fluid was found to be lacking in tyrosin, whereas the tyrosinase was as active as in normal larvae and acted readily on commercial tyrosin with melanin formation. In periods from seven hours to twenty-eight hours, the results were usually similar, but there appeared also to be a gradual reduction of the amount of active tyrosinase. Thus, in one experiment, after twenty-seven hours, no tyrosin could be demonstrated and the amount of tyrosinase was notably reduced.

It has thus been shown that one definite effect on larvae of oxygen deprivation is that their haemocoele fluid loses its power of forming melanin when exposed to air. After short periods of oxygen starvation, this result is due to the absence of tyrosin from the fluid ; after longer periods there also appears to be a reduction in the tyrosinase, as well as absence of tyrosin. In certain cases, after prolonged exposure, while the tyrosinase could not be demonstrated, there was tyrosin present, possibly an evidence of a re-accumulation of tyrosin. That the larva is capable of regenerating a haemocoele fluid apparently normally rich in tyrosinase and tyrosin, is shown by the following experiments.

Larvae which had been exposed to a nitrogen atmosphere, were tapped of all their available haemocoele fluid ; this failed to blacken owing to the absence either of tyrosinase or of tyrosin. On leaving such larvae exposed to air, they recovered and when after an interval they were tapped again, the new haemocoele fluid which they had produced, blackened normally. Similarly, five third instar larvae were exposed to nitrogen for sixteen hours, and then allowed to

recover in air for six hours ; thereafter, the haemocoele fluid was tapped off and blackened normally.

We have already shown that the haemocoele fluid of oxygen-starved larvae was incapable of forming melanin. It remains to be seen if such a fluid which cannot form melanin is still capable of taking up oxygen from the air. If it is capable of doing so, it would tend to show that haemocoele fluid can store oxygen without melanin production resulting and it might be legitimate to infer that it is from this source that the larvae derives the oxygen necessary for its survival for prolonged periods in an atmosphere devoid of oxygen.

In the experiments, third instar larvae were exposed to an oxygen-free atmosphere for respectively three, four and a half, and twenty-six and a half hours. At the end of these periods of anaerobiosis, the larvae were chloroformed. Their haemocoele fluid was pipetted off and placed in a closed ampoule connected with a manometer. Although in accordance with our previous results, no blackening occurred in the haemocoele fluids, oxygen was absorbed. In this case the amount of oxygen taken up was inversely proportional to the time of exposure of the larvae to nitrogen. In these experiments the following figures were obtained :—

(1) The haemocoele fluid of three larvae oxygen starved for twenty-six hours took up 0·05 c.c. of oxygen in twenty hours.

(2) The fluid of three larvae oxygen starved for four and a half hours took up 0·22 c.c. of oxygen.

(3) The fluid of two larvae oxygen starved for three hours took up 0·17 c.c. of oxygen.

Barnes and Grove (1916) made a large number of experiments with living rice weevils, in an apparatus by means of which the insects could be subjected to an atmosphere consisting solely of a single gas ; the gases used were carbon dioxide, hydrogen and nitrogen. The apparatus was so arranged that samples of the gas could be removed for analysis at any time during the experiment. It was anticipated that they would thus establish the changes produced in these gases by the insects. The results of the experiments showed that the insects survived long periods of exposure in a pure atmosphere of any of these gases. No change in the composition of the gases was noted at any time as a result of the insects living in

them even for fifty-five hours and longer. The authors, however, discuss their failure to detect gaseous change by means of this apparatus, and say, ' On the other hand, it must be conceded that the amount of gas brought into play by the number of insects experimented on, is probably so small compared with the total amount of gas present in the incubation tube that the analytical methods at our disposal were too crude to detect the minute change, if any, which had taken place.'

They then made experiments, using the same apparatus, but with large numbers of live larvae instead of a few adults. Thus, instead of ten adults, they used a bulk of 50 grammes of live larvae. They were able to demonstrate the fact that the larvae practically exhausted the atmospheric oxygen, bringing it down from the normal to about 1 per cent. ; the larvae never reduced the oxygen to a lower figure than this. The larvae, however, continued to produce carbon dioxide, after the oxygen had been reduced to the basic figure of 1 per cent. At the end of three or four days, the carbon dioxide concentration had increased by 1·6 per cent. The authors consider that the continued survival of the larvae with the accompanying production of carbon dioxide in an oxygen-free atmosphere, must be due to enzymic action. As proof of this they incubated sterile crushed larvae in a limited supply of air and found, exactly as in the case of the living larvae starved of oxygen, that a process of respiration went on, resulting in the production of large amounts of carbon dioxide. They provided two controls heated with steam in the autoclave, the first for twenty minutes at 115° C., the second for thirty minutes at 125° C. The fact that in the first control the carbon dioxide production was greatly reduced and in the second almost negligible, is cited by them as proof that the respiratory action in sterile crushed larvae is due to enzyme action. According to their theory, the larva when deprived of atmospheric oxygen, ' must be making use of chemically combined oxygen to maintain the life processes during the period of coma which precedes death.' They show that the amount of fat present in larvae starved by limitation of oxygen for five days is 2 per cent. less than that of the normal larvae. They regard this fat as the source of the oxygen utilized by the larva when atmospheric oxygen falls. Since the only enzyme specifically mentioned by them in this connection is tyro-

sinase, this enzyme is presumably the one which they regard as the active agent responsible for supplying the larva with the oxygen stored in the fats.

We did not become aware of the interesting and important work by Barnes and Grove until such time as our own experiments had led us to conclude that the enzyme tyrosinase played an important part in the survival of the insect during prolonged deprivation of atmospheric oxygen.

Our experiments have extended considerably beyond the object of our original investigation regarding the power of the Cordylobia larva to survive occlusion of its spiracles in immune and semi-immune animals. This extension has enabled us not only to confirm the original work of Barnes and Grove, but also to establish certain other facts, which appear to us to be of importance in the physiology of respiration not only of Cordylobia, but probably also of certain other insects. Our findings for Cordylobia larvae are in agreement with those of Barnes and Grove for the Indian rice weevils in that :— Cordylobia larvae can survive long periods of deprivation of atmospheric oxygen; when thus deprived of atmospheric oxygen, they continue for long periods to produce carbon dioxide ; in an ordinary atmosphere the tissues of freshly-killed larvae take up oxygen and give off carbon dioxide for many hours ; in an oxygen-free atmosphere, such tissues continue to give off carbon dioxide ; the substance which enables the larva to live in and produce carbon dioxide in an oxygen-free atmosphere is an enzyme, which also is responsible for the power of freshly-killed larval tissues to take up oxygen and give off carbon dioxide. Our experiments show further :—

(1) That the enzyme tyrosinase and its substrate tyrosin are, in the larva of Cordylobia, confined to the haemocoele fluid.

(2) That the haemocoele fluid taken from a normal larva and exposed to air takes up oxygen and gives off carbon dioxide for prolonged periods after the maximum blackening has occurred.

(3) That the haemocoele fluid, when placed in a nitrogen atmosphere, is still capable of giving off carbon dioxide.

(4) That when the living larva is exposed for a sufficient time to an atmosphere of pure nitrogen, its haemocoele fluid undergoes a change in composition, so that it loses its power of blackening on exposure to air owing in early

stages to the absence of tyrosin, in the later ones to the absence of tyrosinase.

(5) That such altered haemocoele fluid, although unable to blacken normally, is still capable in an ordinary atmosphere of taking up oxygen.

(6) That the power of resisting oxygen deprivation which is so great in the third instar larva and is present to a less extent in the first instar larva is much less marked in the fly.

It is seen from the above facts that the larval haemocoele fluid by itself possesses the properties of oxygen absorption and carbon dioxide production, properties attributed by Barnes and Grove to the larval tissues in general.

V. EXCRETA.

The quantity of faeces passed by a larva during its sojourn in the host is difficult to estimate, but the amount must be very large. *In vitro* it was found that thirteen third instar larvae produced 4½ minims of faeces in four hours. The reaction of samples of the faeces thus passed was found to vary from 7·3 to 8·5, and the colour varied from very light amber to almost black.

Toxicity. The excreta of third instar larvae were found to be highly toxic when injected cutaneously into normal guinea-pigs, but this toxicity was much less marked in animals with a previous history of Cordylobia infection. Three animals died within two days of the intracutaneous injection of from 0·005 c.c. to 0·015 c.c. of larval excreta preserved with chloroform. When the faeces were preserved in glycerine for a short period, the dose of 0·005 c.c. was again fatal in two days in the case of one animal. On halving the dose, no fatal result occurred in three animals. The reaction to the subsequent injections became progressively less marked, so that three times the lethal dose was borne without marked reaction.

Haemolysins, agglutinins and *coagulins* could not be demonstrated, but a powerful anti-coagulin was found to occur in the excreta of the larva.

Precipitins. We have been able to demonstrate the presence of precipitating substances in the faeces. Our first experiments, carried out with the same dilution as used for the haemocoele fluid, i.e., 1-100, revealed its presence, but not constantly, owing, as was later

discovered, to the concentration of the test antigen being too great. The precipitin results obtained with 1-100 faeces were not so well marked nor so constant, as were those obtained with haemocoele fluid 1-100. It was found, however, that on diluting the excreta, constant positive results were obtained with the sera of animals previously infected by Cordylobia. The excreta of the larvae gave a precipitin reaction up to a dilution of 1-1,600 with the serum of guinea-pigs on which larvae had developed previously, and 1-400 was found to be the optimum dilution for the excreta. This was used as our standard dilution while, as we have already stated, in the case of haemocoele fluid, 1-100 was the standard dilution used for testing the serum of the same animals.

We have shown that the serum of animals on which larvae have previously developed, invariably gives a positive precipitin reaction with the haemocoele fluid and excreta of third instar larvae, and that the precipitinogen of the faeces is present in more concentrated form than that of the haemocoele fluid. It remains to be shown to which of these substances the animal first develops a precipitin in its blood. So far as our experiments have gone, we may say that when larvae are growing upon guinea-pigs, the precipitin to the faecal antigen tends to develop before that to the haemocoele fluid antigen. Some of the following animals in Table III illustrate this fact :—

TABLE III.

Showing the results of tests with haemocoele fluid and excreta.

Animal number	Before larvae applied		After application of larvae			
			Test number 1		Test number 2	
	H. fluid	Excreta	H. fluid	Excreta	H. fluid	Excreta
1	–	–	–	+	+	*tr.
2	–	–	–	tr.	–	++
3	–	–	–	–	+	·+÷
4	–	–	+	+	+	+
5	–	–	++	++	++	+.++

- = Negative. + = Positive.
' This test was done a month later than test No. 1.

One guinea-pig developed precipitin to both haemocoele fluid and faeces twelve days after the application of larvae, while another guinea-pig in six days developed precipitin to faeces but not to haemocoele fluid.

The precipitating substances of the haemocoele fluid and the faeces.

We attempted to determine whether these were the same substances or not, by two sets of experiments : (1) On treating serum, which gave a positive reaction with both haemocoele fluid and faeces, with haemocoele fluid, 1 vol. to 20 vols. of serum, and incubating for two and a half hours, at 37° C., it was found that the precipitin to the haemocoele fluid fell greatly, while that to the faeces remained at the same level. Repetition of the absorption caused the precipitin to disappear almost entirely as far as the haemocoele fluid was concerned, but in this case there was some diminution in the faeces precipitin also. In the same way, serum which gave reactions with both haemocoele fluid and faeces, was treated with faeces and incubated as above, it was then tested with haemocoele fluid and faeces. The precipitin with the faeces was found to have fallen to a mere trace, whereas the haemocoele fluid gave a + + reaction ; (2) Animals were injected with haemocoele fluid alone and their serum, after a period, was tested for precipitin with haemocoele fluid and faeces. It was found that a precipitin was obtained early with haemocoele fluid, which rapidly reached a + + + level, at a time when the faeces gave no trace of precipitin. When tested a fortnight later, the faeces also gave a positive result ; in the meantime, several injections of haemocoele fluid had been given. Animals were injected with faeces alone and after an interval their serum was tested with haemocoele fluid and faeces. Precipitin with faeces appeared very rapidly, being slightly positive in six days and increased to a + + + level by the eleventh day. During this time the haemocoele fluid tests were negative ; when tested again, six days later, i.e., seventeen days after the original injection, it was found that the faeces still gave a strong positive reaction, but that now the haemocoele fluid gave a doubtful trace. Further injections of the faeces had meantime been given to the animal.

From these two sets of experiments we conclude that the faeces contain a precipitinogen not present in the haemocoele fluid and

that the haemocoele fluid contains a precipitinogen peculiar to itself, while there is a trace of some substance common to both.

It appears that for diagnostic purposes the precipitin reaction with the faeces, appearing earlier and with a higher titre, has some advantages over that with the haemocoele fluid. We have found that the faeces retains its precipitating power for at least a month when kept in the ice chest with chloroform added to it ; it is probable that it retains its power for longer periods.

Eosinophilia.

In our previous paper we recorded that eosinophilia follows the development of Cordylobia larvae on guinea-pigs, and also the cutaneous injection of ground-up third instar larvae ; that the injection of salivary glands, cuticle, or intestinal tract failed to produce recognisable eosinophilia if the original percentage of eosinophils in the blood of the animal was about one or lower. These portions of the larva were, unfortunately, not tested on animals having a count above 1 per cent. In the case of haemocoele fluid, we have here shown that this is capable, when injected cutaneously into an animal with an eosinophil count of 2·9 per cent., of producing a marked rise in its eosinophil percentage. We have found that faeces also sometimes markedly increase the eosinophil percentage when injected cutaneously into animals. In one of these faeces injection experiments we were able for the first time to raise the eosinophil percentage from a figure well below 1 ; this fact is shown below.

	Day																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	18	20
Injection ...	+	...	+	...	...	+	...	+	...	...	...	...	...	...	...	...	...
Eosinophil percentage	0·4	0	0·5	0·2	0·1	0	0·2	0·7	0	0·9	0·7	0·7	0·8	0·3	1·3	2·0	2·5

It is probable that the eosinophilia which follows the natural development of larvae on an animal, is due to the excreta ; since

the injection of excreta produces per unit amount injected a greater rise in the eosinophil percentage than does the injection of haemocoele fluid.

Immunising properties of the excreta.

We have already shown that in immune animals the penetration of the first instar larva into the skin is followed by the death of about 80 per cent. of the larva within forty hours ; we recorded that many of the larvae which died in the skin of the immune animal in this manner had their posterior end covered by an adherent cap consisting of semi-disintegrated cells lying in a homogeneous matrix. We have investigated this phenomenon much more fully, especially during the first twenty-four hours, i.e., before the death of the larvae in the immune animal, and have found that while only about 50 per cent. of such larvae extracted from an immune animal after twenty-four hours showed a definite cap, all showed the presence of a loosely adherent whitish coagulum surrounding them and giving the larva a woolly appearance. Larvae removed from normal animals rarely showed more than a trace of such a coagulum and then sparsely and irregularly distributed. In addition to this confirmation of our previous findings, we have now demonstrated the fact that all larvae extracted from the skin of immune guinea-pigs have their gut distended with a mass of fine granules, a condition not found in larvae similarly extracted from non-immune animals.

The main points of difference observed between larvae at twenty-four to forty-eight hours in an immune animal and a normal animal are as follows :—

	Non-immune	Immune
1	Extracted with difficulty.	Easily extracted.
2	Alive and active.	Dead or feebly moving.
3	Obviously increased in size.	No increase in size, sometimes shrunken.
4	No adherent coagulum.	Adherent coagulum.
5	Gut normal in size and appearance, contents usually very translucent.	Gut distended, sometimes enormously so, with fine opaque granules.

Some of these points are shown in the illustrations (fig. 6) below.

We endeavoured to imitate *in vitro* the sequence of events which led to the death of larvae in an immune animal. First instar larvae from one to five days old, taken from the sand in the breeding cages, were immersed in sera obtained from immune and non-immune guinea-pigs and examined at intervals. No precipitate formed
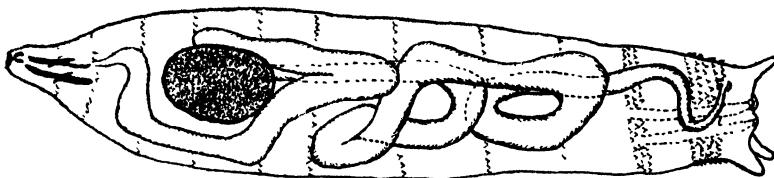
FIG. 6a. First instar larva after 24 hours in non-immune guinea-pig. Length 1·31 mm.
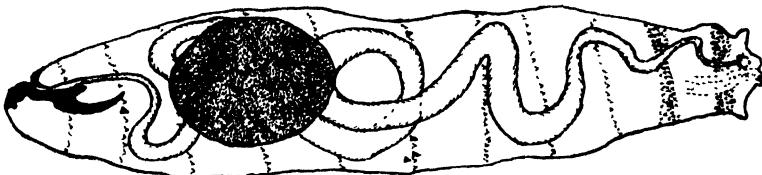
FIG. 6b. First instar larva after 48 hours in non-immune guinea-pig Length 1·52 mm.
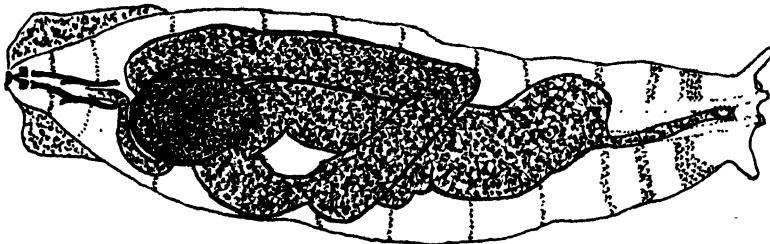
FIG. 6c. First instar larva after 48 hours in guinea-pig (immune). Length 1·19 mm.

around the larvae within a period of about two days' observation ; and as we stated in our previous paper, the immune serum had no more lethal effect than the non-immune serum. When first instar larvae which had been allowed to develop for twenty-four hours in a non-immune animal, were similarly tested, the results were

strikingly different   When such larvae were immersed in the non-immune serum, no precipitate formed round them for a considerable period, but when similar larvae were immersed in immune serum, a precipitate quickly formed round them and this precipitate gradually increased in density.   We came to the conclusion that this difference noted after immersion in immune serum between larvae from sand and larvae, of similar age, from an animal, might be due to the fact that larvae free-living on the sand were obtaining no food and had the gut empty, whereas larvae on the animal were obtaining food and were excreting.   The excreta, as we have shown, give a positive precipitin reaction with immune serum and we therefore thought it highly probable that the coagulum invariably found on larvae extracted from the skin of immune animals is the result of the interaction of the antibody in the animal's tissues and the antigen present in the excreta of the larva.   This precipitin would also explain the distension of the gut by fine granules which we have observed in larvae extracted from immune animals.

In support of this view, we have observed that the clear exudate from a cavity left by a larva in a non-immune animal, produces a marked precipitate when mixed with immune serum.   During the course of the precipitin tests it was observed that two guinea-pigs lost their immunity.   At the same time that these animals lost their immunity it was noted that their precipitin reaction with larval excreta became markedly reduced.   In both of the animals the haemocoele fluid precipitin remained high.   These facts are shown in tabular form below.

Precipitin reaction with	Haemocoele fluid		Excreta	
	Before breakdown of immunity	During breakdown of immunity	Before breakdown of immunity	During breakdown of immunity
Guinea-pig 1 ...	+++	++	+++	tr.
Guinea-pig 2 ...	+++	++	+++	tr.

A third animal in which immunity broke down also showed a definite diminution in the precipitin reaction with the excreta, but in this case the action with the haemocoele fluid was also reduced.

We may briefly summarise the facts which we have ascertained from our experiments as regards the association of the larval excreta and immunity.

1. The serum of guinea-pigs on which larvae have developed naturally, always gives a precipitin reaction with the larval excreta.

2. The excreta of larvae give a strong precipitin reaction with the serum of immune animals so long as they retain their immunity.

3. The injection of larval excreta into normal guinea-pigs always results in the production of a specific precipitin.

4. Local animals which give a negative precipitin reaction always allow the development of a greater proportion of larvae than local animals which give a positive reaction.

5. Larvae recovered from immune animals before the immune animal has had time to destroy them always show a precipitate around them. Larvae removed from non-immune animals very rarely show any such precipitate and where they do it is trivial in amount.

6 Larvae recovered from immune animals always have their gut distended with fine granules. Larvae of a similar age recovered from previously uninfected animals do not exhibit this condition.

7. Larvae from non-immune animals when removed alive and immersed in immune serum invariably develop a precipitate round them, whereas when such larvae are placed in non-immune serum, no precipitate forms.

8. Fluid taken from the cavity previously occupied by the larva similarly gives a precipitate with immune serum, but not with non-immune serum.

9. When immunity breaks down from any cause the precipitin reaction with the excreta becomes markedly diminished.

So far as our experiments have gone, therefore, we have produced a considerable amount of evidence to show that the precipitate which is always formed in the gut of, and round, the larva in immune animals (fig. 7) is due to the interaction of the animal's serum with the excreta of the larva. It seems reasonable to suppose that the apparent blocking of the gut and the enveloping precipitate round the cuticle must hamper the normal development of the larva and may be the direct cause of the death of the parasite, in the skin of the immune host. We are at present engaged in carrying out a

series of experiments in order to ascertain whether the excreta of the third instar larva of *Cordylobia anthropophaga* can be used successfully as an antigen with which to produce immunity.
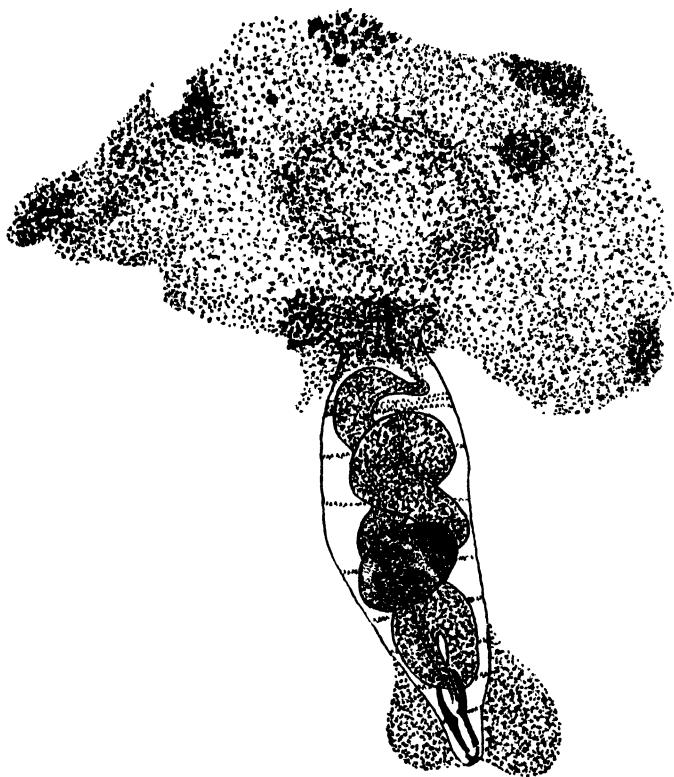
Fig. 7. Composite figure from camera lucida drawings of first instar larva removed after 48 hours from immune guinea-pig. Showing cap over head and tail.

The practical value of the precipitin reaction.

It would appear that we have in this reaction with haemocoele, fluid or excreta a test which should be of great value in the early diagnosis of Hypoderma infections. At present no means of diagnosing Hypoderma infection exists until the full-grown larva appears in the skin of the back about seven or nine months after infection takes place. In connection with this we may quote Bishopp and others (1926), who write in No. 1369 of ' The United States Department of Agriculture Bulletin ' : ' The importance of *H. bovis*

as a cattle pest is sufficient to warrant serious consideration of ways and means of checking its spread in this country. It may not be available to take legal steps to meet the situation, but certainly stock raisers should recognise the danger of bringing cattle infested with *H. bovis* into regions where that pest does not exist, and take due precautions to destroy all grubs which reach the subcutaneous tissues of the backs during the spring and summer following the arrival of the cattle. Certain uninfested foreign countries have enacted legislation designed to prevent the establishment of Hypoderma. Australia, for instance, has a law prohibiting the introduction of cattle from the United States, the British Isles, and other infested countries except during the period from December 1 to May 31. Present knowledge of the seasonal history of Hypoderma shows clearly that such a restriction would not give a complete protection against the introduction of the pests. It would appear, however, that some system by which the animals could be kept under surveillance and all grubs destroyed during the period of one full year after importation would be effective.'

Before the reaction which we have here described can be proved of practical value, it will be necessary to show that it is sufficiently specific to enable us to distinguish Hypoderma infestation from infestation with other parasites such as helminths. So far as Great Britain is concerned, the question of other myiasis in cattle would not lead to confusion unless a double infection exists. Even then it should not occur because we have been able to distinguish by the sharpness of the reaction the coelomic fluids of such closely allied insect parasites as Cordylobia and Sarcophaga. One of the experiments is given below.

Number of serum	Type of guinea-pig	Haemocoele fluid			
		Cordylobia		Sarcophaga	
		5 mins. at room temp.	15 mins. at 37° C.	5 mins. at room temp.	15 mins. at 37° C.
1	Non-immune ...	0	0	0	0
2	Immune No. 1	+	++	0	+
3	Immune No. 2	++	+++	0	++

It being apparently possible to distinguish by the precipitin reaction parasitism by two closely allied insects, we should not anticipate that the serum of animals harbouring helminthic parasites would on that account cause confusing positive precipitin reactions when using Hypoderma antigen.

SUMMARY

1. Injections of whole first instar *Cordylobia anthropophaga* larvae and antigens prepared from the cuticle, salivary glands and gut of third instar larvae have only produced immunity in a few isolated instances and then only after the injection of relatively very large amounts of such antigens. The toxicity of these antigens and the development of immunity to such toxins has been studied.

2. The following enzymes have been found to occur in the third instar larva :—Amylase, invertase, maltase, trypsin, erepsin, lipase and tyrosinase ; an account is given of their distribution and relative concentration.

3. Immune and non-immune guinea-pigs' sera were found to possess both amylase and lipase. In a few experiments the presence of an invertase accelerator was also demonstrated.

4 Neither immune nor non-immune animals showed any anti-enzyme to amylase, invertase, or lipase. Both immune and non-immune animals possessed to an equal extent anti-trypsin and anti-tyrosinase.

5. Injection of amylase, invertase, and lipase into guinea-pigs was not followed by the development of an anti-enzyme. Injection of tyrosinase (i.e., the haemocoele fluid of third instar larvae containing both tyrosinase and tyrosin) into guinea-pigs did not increase the already existing anti-tyrosinase.

6. Injection of trypsin into guinea-pigs was followed by the further development of anti-trypsin, but animals which had acquired this antibody possessed no immunity to Cordylobia larvae.

7. The power possessed by third instar larvae of resisting long periods of oxygen deprivation has been studied. We have reached the conclusion that this power is dependent on the enzyme tyrosinase

present in the haemocoele fluid of the larva. Our experiments have confirmed and amplified the work of Barnes and Grove (1916).

8. Eosinophilia followed the injection of the haemocoele fluid or excreta of third instar larvae into guinea-pigs. A similar result did not follow the injection of whole first instar larvae, or of antigens prepared from the cuticle, salivary glands or gut of third instar larvae.

9. No precipitin in the serum of previously infected animals could be demonstrated to antigens composed of ground first instar larvae or of cuticle, salivary glands or gut of third instar larvae. The injection of large amounts of these antigens only rarely led to the development of a weak specific precipitin.

10. A precipitin has been shown to exist in the serum of previously infected animals to the haemocoele fluid and excreta of third instar larvae. No such precipitin was ever demonstrated in the serum of animals which had not been infected.

11. The death of the larva in the immune animal has been shown to be very closely associated with the reaction between the gut contents of the larva and the serum of the immune animal.

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BACTERIOLOGICAL INVESTIGATION ON *CORDYLOBIA ANTHROPOPHAGA* IN RELATION TO THE IMMUNITY PRODUCED BY IT

BY

MARION WATSON

In papers previous to the one of which this bacteriological investigation forms a part, Blacklock and Thompson (1923), and Blacklock and Gordon (1927) have shown that an immunity to the attack of the larvae of *Cordylobia anthropophaga* can be produced experimentally in guinea-pigs as the result of repeated infection by the larvae. The factor responsible for the production of the immunity is still unknown and the investigation detailed below was undertaken in an attempt to ascertain what part, if any, bacteria play in the production of the immunity. The work done has been mainly of a preliminary character, and the experiments carried out do little more than pave the way for future work, but it was evident that such preliminary work was necessary. From the literature available for reference no information could be got regarding the normal bacterial flora of the skin of guinea-pigs and other common hosts of *C. anthropophaga* larvae, and as the site of election of the larvae is the superficial layers of the skin, it seemed desirable that the foremost step should be to find out what organisms normally were present in such skins. The first part of this paper, therefore, deals first with the organisms isolated from the skins of two groups of guinea-pigs, (*a*) those with normal skins infected with *C. anthropophaga* larvae, and (*b*) those whose skins, as the result of repeated experimental infection, were immune to further attack by the larvae ; and secondly, with the bacteria isolated from the skin of rats. The second part of the paper deals with the identification of the bacterial flora of the parasite itself at its various stages of growth, the material for examination consisting of whole flies or larvae, or of their intestinal contents.

In this first part of the investigation, it was thought desirable to get an all-round view of the situation before concentrating on any one point, and in consequence the numbers in each series of experiments are small. For this reason the results, although they show a considerable degree of uniformity, are open to criticism.

Many of the organisms isolated are still unidentified but it is hoped that the majority of these may be identified later. It was only after the completion of the bacteriological examination of the larvae that the work of Graham Smith (1913) became available for reference, and it is proposed, at a later date, to classify, more in accordance with his grouping, the non-lactose fermenting, non-gelatin liquefying organisms isolated from the intestinal tracts of flies and larvae.

Organisms cultivated from the skin of guinea-pigs.

The skins of ten guinea-pigs were examined. Five of these guinea-pigs were imported English guinea-pigs whose skins, as a result of repeated experimental infection, had been rendered immune to further infection by the larvae, and five were imported English guinea-pigs which had received no experimental infection. The method of obtaining skin for examination was the same for all the guinea-pigs, and was as follows :

Without any preliminary sterilization an area of the abdomen of each guinea-pig was dry-shaved and a piece of skin, measuring 3 to 4 mm. in diameter, was removed with sterile scissors and forceps, care being taken to remove only the superficial layer of skin and to avoid bleeding. Each piece of skin after removal was stroked on ordinary agar and McConkey agar plates, and then was placed in a test-tube containing nutrient broth.

Another portion of the shaved area of skin of each guinea-pig's abdomen was then cleaned with iodine and spirit, to kill surface organisms, and the procedure repeated.

Colonies of various types were picked off the agar and McConkey agar after 24 hours' and 48 hours' incubation under aerobic conditions at 37° C., and replated. The broth cultures, incubated aerobically at 37° C., were plated on agar daily for 7 days, and from these plates colonies were picked off and replated, and replating was continued until pure cultures were obtained. The large number of spore-bearing organisms present made the separation of organisms in pure culture difficult, and in the case of some organisms replating over a period of several months was necessary before pure cultures were got. Contamination of plates was rapid, and because of this colonies appearing later than 48 hours' incubation were ignored unless their number and distribution on the plate justified the

assumption that their presence was not due to contamination. The appearance of moulds on the plates was frequent, but no examination of these was made. Each organism, when obtained in pure culture, was investigated fully as to morphology and cultural characteristics.

From the ten guinea-pigs, 128 organisms were isolated, but 15 of these were found to be present in duplicate form in the same animals, and, although fully examined, have been omitted from the following tables. The distribution of the remaining 113 organisms among the ten guinea-pigs is given in Tables I and II.

TABLE I.

Showing the number of organisms isolated from the skin of normal guinea-pigs.

	Skin dry shaved	Skin dry shaved and cleaned
Normal Guinea-pig 1	9 organisms	3 organisms
Normal Guinea-pig 2	12 ,,	4 ,,
Normal Guinea-pig 3	5 ,,	3 ,,
Normal Guinea-pig 4	11 ,,	4 ,,
Normal Guinea-pig 5	6 ,,	3 ,,
	43 ,,	17 ,,

TABLE II.

Showing the number of organisms isolated from the skin of immune guinea-pigs.

	Skin dry shaved	Skin dry shaved and cleaned
Immune Guinea-pig 1	6 organisms	3 organisms
Immune Guinea-pig 2	10 ,,	4 ,,
Immune Guinea-pig 3	5 ,,	2 ,,
Immune Guinea-pig 4	9 ,,	3 ,,
Immune Guinea-pig 5	8 ,,	3 ,,
	38 ,,	15 ,,

Seventy-eight of the 113 organisms have been identified, and the distribution of these 78 organisms among the ten guinea-pigs is shown in Table III.

TABLE III.

Showing the distribution of various identified skin organisms among normal and immune guinea-pigs.

Organisms	Normal Guinea-pigs											Immune Guinea-pigs										
	1		2		3		4		5		Total	1		2		3		4		5		Total
	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin		Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	
Actino. graminis, Bostroem ...	+	−	−	−	−	−	−	−	−	−	1	−	−	−	−	−	−	−	−	−	−	0
Streptotbrix ...	−	−	−	−	+	+	−	−	−	−	2	−	−	−	−	−	−	−	−	−	−	0
C. psuedo-tuberculosis ...	−	−	−	−	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	+	−	1
Str. faecalis ...	−	−	−	−	+	−	−	−	−	−	1	−	−	−	+	−	−	−	−	−	−	1
Sta. aureus ...	−	−	−	−	−	−	+	−	−	−	1	−	−	−	−	−	−	−	−	−	−	0
Sta. albus	+	−	−	−	−	−	+	+	−	−	3	+	−	−	−	+	−	−	+	−	−	3
Micro. tetragenus ...	−	−	−	−	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	+	−	1
Sarcina lutea ...	−	−	+	−	−	−	−	−	−	−	1	−	−	−	+	−	−	−	−	−	−	1
Sarcina ventriculi	−	−	−	−	−	−	−	−	−	−	0	−	−	−	−	−	−	+	−	−	−	1
Cbr. prodigiosum ...	−	−	+	−	−	−	−	−	−	−	1	−	−	−	−	−	−	−	−	−	−	0
Cbr. aquatilis ...	−	−	−	−	−	−	−	−	−	−	0	+	−	−	−	−	−	−	−	−	−	1
Bact. coli	+	−	−	−	+	−	−	−	+	−	3	+	−	−	−	+	−	+	−	+	−	4
Past. pseudo-tuberculosis ...	−	−	+	−	−	−	−	−	−	−	1	−	−	−	−	+	−	−	−	−	−	1
B. subtilis	+	+	+	+	−	−	+	+	+	+	8	−	−	+	+	−	−	+	+	+	+	6
B. megatherium ...	+	+	−	−	−	−	−	−	+	+	4	−	−	−	−	−	−	+	+	+	+	4
B. mesentericus viscosus ...	−	−	−	−	+	+	+	+	−	−	4	−	−	+	+	−	−	−	−	+	+	4
B. mesentericus fuscus ...	−	−	+	+	−	−	+	+	−	−	4	−	−	−	−	−	−	+	−	−	−	1
B. mesentericus vulgatus ...	−	−	+	+	−	−	−	−	+	+	4	+	+	−	−	−	−	−	−	−	−	2
B. mycoides ...	−	+	−	−	+	+	−	−	−	−	3	+	+	−	−	+	+	−	−	−	−	4
B. anthracoides ...	−	−	−	−	−	−	−	−	−	−	0	−	−	−	−	+	+	−	−	−	−	2
Totals ...	5	3	6	3	5	3	5	4	4	3	41	5	2	4	2	5	2	5	3	6	3	37

Of the 35 organisms not yet identified, in the normal skin group 2 were cocco-bacilli, 4 were non-sporing bacilli, and 13 were sporing bacilli; in the immune skin group 3 were cocci, 3 were cocco-bacilli, 4 were non-sporing bacilli, and 6 were sporing bacilli. Of the non-sporing bacilli, 3 in the normal skin group and 4 in the immune skin group were coliform-like organisms resembling those isolated later from rat skins, flies, and larvae. Tables IV and V show the distribution of these unidentified organisms.

TABLE IV.

Showing distribution of various unidentified skin organisms among normal guinea-pigs.

| Organisms | Normal Guinea-pigs | | | | | | | | | | Total |
| | 1 | | 2 | | 3 | | 4 | | 5 | | |
	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	
Cocci	...	...	...	...	...	...	...	...	...	...	o
Cocco-bacilli ...	...	...	1	...	...	...	1	...	...	...	2
Bacilli	1	...	1	...	...	...	1	...	1	...	4
Sporing bacilli ...	3	...	4	1	...	...	4	...	1	...	13
Totals ...	4	...	6	1	...	...	6	...	2	...	19

TABLE V.

Showing distribution of various unidentified skin organisms among immune guinea-pigs.

| Organisms | Immune Guinea-pigs | | | | | | | | | | Total |
| | 1 | | 2 | | 3 | | 4 | | 5 | | |
	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	
Cocci	...	...	1	1	...	...	...	...	1	...	3
Cocco-bacilli ...	...	...	1	...	...	...	1	...	1	...	3
Bacilli	1	1	1	1	...	...	...	...	...	...	4
Sporing bacilli ...	...	...	3	...	...	...	3	...	...	...	6
Totals ...	1	1	6	2	...	...	4	...	2	...	16

Organisms cultivated from skins of rats.

The skins of ten local wild rats were examined, the technique throughout being the same as that employed in the examination of the guinea-pigs' skins. Some of the skins examined showed evidence of·infection with *C. anthropophaga* larvae, and some appeared free from infection. It is probable, however, as the incidence of infection among local wild rats is high, that those rats which showed no evidence of infection at the time of examination may have had one or more previous infections.

From the ten rats, 58 organisms were isolated, and their distribution among the rats is shown in Table VI.

TABLE VI.

Showing number of organisms isolated from the skin of rats.

	Skin dry shaved	Skin dry shaved and cleaned
Rat 1	5 organisms	3 organisms
Rat 2	5 ,,	2 ,,
Rat 3	4 ,,	2 ,,
Rat 4	4 ,,	2 ,,
Rat 5	3 ,,	2 ,,
Rat 6	4 ,,	2 ,,
Rat 7	4 ,,	1 ,,
Rat 8	4 ,,	1 ,,
Rat 9	3 ,,	1 ,,
Rat 10	4 ,,	2 ,,
	40 ,,	18 ,,

Forty-five of the 58 organisms have been identified, and Table VII shows their distribution among the ten rats.

TABLE VII.

Showing distribution of identified skin organisms among ten rats.

Organisms	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Rat 6		Rat 7		Rat 8		Rat 9		Rat 10		Total
	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	Normal skin	Cleaned skin	
Str. faecalis	+	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	3
Sta. albus	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	2
Sarcina lutea	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2
Ps. pyocyanea	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	2
Proteus vulgaris	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	3
Bact. coli	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	4
Coliform-like organism 1	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	4
Coliform-like organism 2	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	3
Coliform-like organism 3	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1
B. subtilis	−	−	+	+	−	−	+	+	−	−	+	+	+	−	+	+	−	−	−	−	9
B. megatherium	−	−	−	−	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	4
B. mesentericus vulgatus	+	+	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	+	+	+	8
Totals	4	2	5	2	2	1	2	1	3	2	2	2	3	0	4	1	2	1	4	2	45

Thirteen organisms have not been identified. Of these, 2 are cocci and 11 are sporing bacilli. Their distribution among the rats is shown in Table VIII.

TABLE VIII.

Showing distribution of unidentified organisms among ten rats.

Organisms	Rat 1 Normal skin	Rat 1 Cleaned skin	Rat 2 Normal skin	Rat 2 Cleaned skin	Rat 3 Normal skin	Rat 3 Cleaned skin	Rat 4 Normal skin	Rat 4 Cleaned skin	Rat 5 Normal skin	Rat 5 Cleaned skin	Rat 6 Normal skin	Rat 6 Cleaned skin	Rat 7 Normal skin	Rat 7 Cleaned skin	Rat 8 Normal skin	Rat 8 Cleaned skin	Rat 9 Normal skin	Rat 9 Cleaned skin	Rat 10 Normal skin	Rat 10 Cleaned skin	Total
Cocci	...	...	...	...	...	...	1	1	...	...	...	...	...	...	...	...	...	...	...	...	2
Sporing bacilli ...	1	1	...	...	2	1	1	...	...	...	2	...	1	1	...	...	1	...	...	...	11
Totals ...	1	1	0	0	2	1	2	1	0	0	2	0	1	1	0	0	1	0	0	0	13

Examination of newly-hatched flies.

Thirteen pupae, taken from the stock used for breeding purposes, were placed in 50 per cent. alcohol for 20 minutes. They were then removed to sterile test-tubes, and the alcohol allowed to evaporate. When free from alcohol, each pupa was dipped in a test-tube containing nutrient broth, to test the sterility of the exterior of the pupa, and then replaced in a sterile test-tube. No growth was obtained from any of the 13 tubes of broth thus treated. From the 13 pupae, 11 flies hatched out. One of these flies escaped, but each of the remaining 10, after hatching from the pupa, was removed to a test-tube of nutrient broth and broken up. All 10 broth tubes showed a heavy growth after 24 hours' aerobic incubation at 37° C., and the cultures were then plated out on agar and McConkey agar. Table IX below shows the organisms isolated and their distribution.

At the time when the pupae were collected, their source of origin was not noted, and it is possible that more than one pupa came from the same fly. This may in part account for the incidence of such organisms as *Proteus vulgaris* and *Pseudomonas pyocyanea.*

TABLE IX.

Showing distribution of organisms isolated, among ten flies.

Organisms	Fly 1	Fly 2	Fly 3	Fly 4	Fly 5	Fly 6	Fly 7	Fly 8	Fly 9	Fly 10	Total
Proteus vulgaris ...	+	+	−	+	+	+	−	+	+	−	7
Ps. pyocyanea ...	−	+	−	+	−	−	+	−	−	−	3
Str. faecalis ...	−	−	+	−	−	+	+	−	−	−	3
Bact. morgani ...	−	−	+	−	−	−	−	−	−	−	1
Coliform-like Organism 1 ...	+	+	−	+	+	+	+	+	−	+	8
Coliform-like Organism 2 ...	+	−	+	+	+	−	−	−	+	+	6
Coliform-like Organism 3 ...	−	+	−	−	−	−	−	+	+	+	4
Totals ...	3	4	3	4	3	3	3	3	3	3	32

Examination of third instar larvae.

Ten third instar were examined, and in six of these ten material from the rat host was examined also.

Larva 1, after removal from the rat, was washed repeatedly in sterile saline, and after the final washing, a small drop of faeces was pipetted from the anus by means of a sterile Pasteur pipette.

Larva 2 was dipped in absolute alcohol and flamed, and a drop of faeces, mixed with coelomic fluid, pipetted from the rectum. The larva was then dissected and the whole alimentary tract removed and ground up in nutrient broth.

Larvae 3 and 4 were removed with sterile forceps from the rat after cleaning up the wound of entrance with iodine and spirit. The larvae were placed in 50 per cent. spirit for fifteen minutes, and then removed to a sterile Petri dish and the spirit allowed to

evaporate. A drop of faeces was removed from the anus with a sterile pipette.

Larva 5 was treated as larvae 3 and 4, but in addition, immediately after the removal of the larva from the rat, some of the fluid in the wound cavity in the rat was pipetted off and added to nutrient broth. Further, after cleaning the shaved skin with iodine and spirit, a drop of blood was taken from the rat's tail (Rat 148) and cultured in broth. From this blood two organisms were isolated, but their presence was probably due to contamination of the blood from the skin, as a second culture of the same rat's blood was sterile.

From larvae 6, 7, 8, 9 and 10, after preliminary cleaning of the wounds in the rats and sterilization of the exterior of the larvae in 50 per cent. spirit, faeces and fluid from the wound cavities were cultured.

In all the above examinations the material obtained was grown aerobically in broth at 37° C., and the broth plated on plain agar and McConkey agar after 24 hours' and 48 hours' incubation. After a further 24 hours' incubation, colonies were picked off and replated, and replating was continued until it appeared certain that the different organisms had been obtained in pure culture. The morphological and cultural characteristics of each organism was then investigated.

The sterility of the exterior of the larvae after 15 minutes' immersion in 50 per cent. spirit, was tested by allowing the larvae, after removal from and evaporation of the spirit, to crawl over agar plates. From the five larvae thus examined, no organisms other than those isolated later from the faeces of the same larvae were cultivated.

Material Examined.

Third instar larva 1. Faeces.
Third instar larva 2. Faeces and coelomic fluid. Intestinal tract.
Third instar larva 3. Faeces.
Third instar larva 4. Faeces.
Third instar larva 5. Faeces. Fluid from wound cavity in rat 148.
Third instar larva 6. Faeces. Fluid from wound cavity in rat 169.
Third instar larva 7. Faeces. Fluid from wound cavity in rat 166.
Third instar larva 8. Faeces. Fluid from wound cavity in rat 167.
Third instar larva 9. Faeces. Fluid from wound cavity in rat 168.
Third instar larva 10. Faeces. Fluid from wound cavity in rat 172.
Rat 148. Blood.

The organisms isolated are shown in Table X.

TABLE X

Showing distribution of organisms isolated, among ten third instar larvae.

Larvae	Bact. morgani	Proteus vulgaris	Coliform-like organism 1	Coliform-like organism 2	Strept. faecalis	Ps. pyo-cyanea	Bact. alkali-genes	Diplo. crassus	Total
Larva 1 Faeces	+	+	-	-	-	-	-	-	2
Larva 2 (a) Faeces and coelomic fluid ...	-	+	+	+	+	+	-	-	5
(b) Intestine	-	+	+	+	+	+	-	-	5
Larva 3 Faeces	-	+	+	+	+	-	-	-	4
Larva 4 Faeces	-	+	+	+	+	-	+	-	5
Larva 5 (a) Faeces	-	+	+	+	-	+	-	+	5
(b) Wound cavity	-	+	+	+	-	-	-	-	3
(c) Rat blood	-	+	-	-	-	-	-	+	2
Larva 6 (a) Faeces	-	+	+	+	-	-	-	-	3
(b) Wound cavity	-	+	+	+	+	-	-	-	4
Larva 7 (a) Faeces	-	+	+	-	+	-	-	-	3
(b) Wound cavity	-	+	+	-	+	-	-	-	3
Larva 8 (a) Faeces	-	+	+	-	+	-	-	-	3
(b) Wound cavity	-	+	+	-	+	-	-	-	3
Larva 9 (a) Faeces	-	+	+	-	+	-	-	-	3
(b) Wound cavity	-	+	+	-	+	-	-	-	3
Larva 10 (a) Faeces	-	+	+	-	-	-	-	-	2
(b) Wound cavity	-	+	+	-	-	-	-	-	2
Totals	1	18	16	8	11	3	1	2	60

Examination of first instar larvae.

In all, 30 first instar larvae were examined. Twenty of these were treated by immersion in 50 per cent. alcohol. They were then allowed to drain on sterile filter paper, and after the alcohol on the skin had evaporated the larvae were ground up in nutrient broth. The twenty broth tubes were incubated at 37° C. for 14 days, and were plated on agar daily. Nineteen of the broth tubes remained sterile, the twentieth showing a growth of *B. subtilis.*

All the 20 larvae were alive when removed from the alcohol, and it was later found that first instar larvae survived one and a half hours' immersion in 50 per cent. spirit.

The skins of the remaining 10 larvae were sterilised by dipping the larvae in absolute alcohol and then burning off the spirit. The larvae were ground up in broth which was incubated at 37° C. for 14 days and plated on agar daily. All ten broth tubes were sterile after 14 days' incubation at 37° C.

Examination of eggs.

Difficulty was experienced in sterilising the exterior of the eggs of *C. anthropophaga* and 15 minutes' immersion in 50 per cent. spirit did not suffice for sterilization. The eggs were surrounded by a sticky gelatinous substance with grains of the sand in which they had been laid adhering to them, and it was only after careful separation of the eggs and immersion in 50 per cent. alcohol for one hour that the exterior of the eggs was found to be sterile. After sterilization the eggs were removed from the spirit one by one, drained on sterile filter paper, placed on sterile agar slopes, and allowed to hatch out. Of a total of 97 eggs, 88 larvae hatched out. After emerging from the eggs, the larvae were removed to tubes of nutrient broth, in which they were ground up. The tubes were incubated at 37° C. for 14 days, and the broth plated out daily on agar. Neither on the agar slopes on which the eggs hatched out, nor from the newly-hatched larvae themselves was any growth got, all the 88 larvae which hatched out proving sterile.

CONCLUSIONS

1.   That in the two groups of guinea-pig skins examined, the bacterial flora of the non-infected skins differed little from that of the immune skins, and further, that no one organism was constantly present and peculiar to one group or the other.

2.   That the bacterial flora of the rat skins examined, although less varied than that of the guinea-pig skins, did not differ materially from the latter.

3.   That the newly emerged *C. anthropophaga*, hatched under sterile conditions, contains organisms similar to those isolated from the intestinal tract of third instar larvae.

4.   That third instar larvae of *C. anthropophaga* have intestinal tracts containing many bacteria, and that the organisms probably are derived largely from the skin or tissues of the host in which they have grown.

5.   That the first instar larvae of *C. anthropophaga*, on emergence from the eggs, are sterile.

6.   That the interior of the eggs of *C. anthropophaga*, laid by flies which have been bred without any precautions to ensure sterility, is sterile.

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THE TRANSMISSION OF MALARIA IN SIERRA LEONE

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EXISTING EVIDENCE

Very little is known regarding the transmission of malaria in Sierra Leone. Below is shown a list of the anophelines which are known to occur there, together with notes on their habits and such records of dissection as we have been able to find cited in the literature.

It is obvious from the work of Covell (1927) and others that, in order to incriminate any species as an actual transmitter of malaria, it is necessary to show that after a bred mosquito has had an infective feed, sporozoites subsequently appear in its salivary glands. This fact, alone, only proves that the species under consideration is a potential carrier: in order to prove that it infects man in nature and to estimate its importance in this connexion the following additional points should be investigated. (1) Does it readily bite man? (2) Does it frequently occur in human dwelling places? (3) To what extent is it found naturally infected? A minor point mentioned by Swellengrebel, Schüffner and S. de Graaf (1919), is the possibility of the vegetable food of the female interfering with the development of the malaria parasite. Mayne (1928) records that *A. subpictus* (*rossii*) is capable of infection in the salivary glands with sporozoites as a result of feeding on birds suffering from *Plasmodium praecox* : this species of anopheline is already known to be capable of infection with all three forms of human malaria, so that Mayne's observation emphasizes the essential importance of feeding experiments carried out with bred mosquitos.

Anopheles (Myzomyia) domicolus Edwards, 1916.

Only one record of this species is known from Sierra Leone (Blacklock and Evans, 1926).

Anopheles (Myzomyia) funestus Giles, 1900.

This is a common Sierra Leone anopheline. Evidence from the Gambia, Dutton (1902), Zanzibar, Mansfield-Aders (1927), and elsewhere, shows that it occurs frequently in houses and readily bites man. Daniels (1901) fed 57 ' wild ' *A. funestus* on a crescent carrier : 27 became infected, 2 having sporozoites in the salivary glands. Ross, Annett and Austen (1900) fed 5 ' wild ' mosquitos on a quartan carrier ; of two subsequently dissected, one was found infected with zygotes only. Newstead, Dutton and Todd (1907) state that ' Malaria parasites were seen to develop in these mosquitos at Lusambo.' It is evident from the above findings that *A. funestus* is probably a transmitter, but the use of wild mosquitos in the experiments referred to precludes the possibility of certainty. The finding of naturally infected mosquitos in native houses supports the possibility of its being a true natural transmitter. Thus Wood (1915) dissected 100 specimens from habitations in Sierra Leone, and found a sporozoite rate of 11 per cent., and Mansfield-Aders (1927) dissected 1,167 specimens from houses in Zanzibar, and found sporozoites in the salivary glands of 80, or 7 per cent. Lamborn (1925), while in Nyasaland, examined 430 of this species and found 10 infected in the gut, and 2 infected in the salivary glands. Dutton (1902) dissected a mixture of 24 *A. funestus* and *A. gambiae*, and found one gut and one gland infection amongst the *A. funestus* so dissected, though the actual number of this species examined is not stated. Schwetz, Collart and Geernick (1929), in the Belgian Congo, dissected 191 females caught in houses and found 4 per cent. with infection of the salivary glands. Strickland (1929), in Assam, found 19 similarly positive amongst 1,489 dissected.

Anopheles (Myzomyia) gambiae Giles (1902).

This mosquito, which is by far the commonest anopheline in Sierra Leone, has always been regarded as the most important malaria transmitter there. It is frequently found in houses, both

native and European, and readily bites man. The evidence regarding experimental infection is surprisingly scanty ; the only record we are aware of is that of Ross, Annett and Austen (1900), who fed one 'wild' *A. gambiae* on a quartan carrier and found, on dissection, two days later, that it was infected with zygotes. The evidence as regards natural infection is much more abundant ; thus Wood (1915) found sporozoites in the glands of 8 out of 91 examined (8·8 per cent.) in Sierra Leone, and Mansfield-Aders (1927), who dissected 1,833 specimens from houses in Zanzibar found sporozoites in the glands of 134 (7·7 per cent.). Ross, Annett and Austen (1900) dissected 109 mosquitos of this species, and found 27 gut infections ; in 5 of these (4·5 per cent.) the salivary glands were also infected. Dutton (1902), in the Gambia, similarly found 4 gut and 1 gland infection amongst 36 dissected (3 per cent.) ; Dutton and Todd (1906), in the Belgian Congo, dissected 92, none of which proved to be infected. Hill and Hayden (1905) found 13 (14·2 per cent.) infected in both gut and glands, out of 91 dissected in Natal. Lamborn (1925) found 10 gut infections amongst 121 examined. Schwetz (1929) found gland infections in 114 amongst a total of 992 dissected.

It is obvious from these results that though the cumulative evidence that *A. gambiae* is a transmitter is almost conclusive, yet the entire absence of experimental infection of bred mosquitos makes it clear that further experiments are required before the evidence against this important anopheline is complete.

Anopheles (Myzomyia) marshalli Theobald (1905).

Varieties of this species are recorded from Sierra Leone by Evans (1927) ; little is known as to its habits, but it has been recorded from houses by Newstead, Dutton and Todd (1907), and Schwetz (1929). The latter author supplies the first record of natural infection occurring in this species. He dissected 154 *A. marshalli* var. *moucheti* and found 7 (4·5 per cent.) infected in the salivary glands. We have failed to find any record of experimental infection.

Anopheles (Anopheles) mauritianus Dabuty and d'Emmerez (1900).

This mosquito is known to occur in large numbers in parts of Sierra Leone, and Gordon (1929) has noted it as being numerous in a

European house. It has also been found in tents and houses in, Egypt, by Kirkpatrick (1925), while Macgregor (1924) says that it not only goes into the houses, but bites in the open at any time of day or night though insusceptible to infection with malaria.

In the original description of *A. paludis*, a species which was subsequently sunk as a synonym for *A mauritianus*, Theobald (1900) states that Major S. R. Christophers, who sent him the species, wrote in a covering letter, ' This species has been shown on two occasions to contain sporozoites in the salivary glands, though caught about a quarter of a mile from human habitation.' Dissections of wild mosquitos by Dutton and Todd (1906) and Ross (1908) have not shown any infections, and the latter regards it as being of no importance in the transmission of malaria in Mauritius.

Anopheles (Myzomyia) nili Theobald (1904).

This anopheline was found breeding at Daru, Sierra Leone, by Blacklock (1925), who noted that although breeding close to dwelling places, it did not usually enter them. Only one specimen was found in a native house, in which very numerous *A. gambiae* were sheltering, although the breeding places of the latter were at a greater distance than those of *A. nili* Schwetz (1929), however, found 132 *A. nili* females amongst a total of 1,469 female anophelines collected from native houses at Stanleyville, Belgian Congo. Apparently no experimental investigation has been made of its powers of transmitting malaria ; that it is possibly a carrier is shown by Schwetz (1929), who dissected 132 specimens caught in native houses in the Belgian Congo, and found 7, that is 5·3 per cent., infected in the salivary glands ; but in Sierra Leone, at any rate, it would seem, on account of its habits, to be unimportant.

Anopheles (Anopheles) obscurus Grünberg (1905).

From 1912 till the time of publication of Christophers' (1924) memoir, this species was not distinguished from *A. umbrosus*. Existing records as to its presence in Sierra Leone and all relevant observations as to its habits were made between these dates, and much confusion has resulted We shall accept Evans' (1927)

classification, whereby all records of *A. umbrosus* from Africa are considered as *A. obscurus*.

We have two records of this species from Sierra Leone. Blacklock (1925) found it breeding at Daru, and Wood (1915) captured three females in dwelling houses at Kaballa. There are no records of experimental or natural infections except Wood's dissection of one wild mosquito which proved negative

Anopheles (Myzomyia) pharoensis Theobald (1901).

There are many records of this species entering dwelling places. It has been recorded from Sierra Leone, but we have no reason to think that it ever occurs there in large numbers.

Amongst a total of five 'wild' *A. pharoensis* dissected by Dutton (1902 and 1906), in the Gambia and the Congo, one was found infected. Newstead, Dutton and Todd (1907) remark that ' Malaria parasites were seen to develop in this mosquito at Boma,' they give no further details of their experiments, but Bahr (1918), working in Egypt, dissected 36 specimens which had fed on a patient with 600 crescents per c.mm., and only found one mosquito infected ; this insect showed two zygotes in the stomach.

The evidence is at present too scanty to draw any conclusions regarding the part played by *A. pharoensis*, in the transmission of malaria in Sierra Leone, but if future investigations continue to show it as a rare species, this, combined with the apparent difficulty in infecting it, would render it of negligible importance as a transmitter.

Anopheles (Myzomyia) pitchfordi Giles (1904).

The only records we can find of the occurrence of this species in Sierra Leone are those of Wood (1914 and 1915), who found it in the Koinadugu district, where, in four months, he captured seven females. He dissected three of these with negative results.

Anopheles (Myzomyia) rhodesiensis Theobald (1901).

This species is widely distributed in Sierra Leone and in places breeds in very large numbers. There are extensive breeding grounds in the vicinity of Freetown. Wood (1915), in a search of European

and native quarters, at Koinadugu, in Sierra Leone, found that it was very numerous in the months of September and October, the numbers apparently falling off later in the year. Gill (1916) records having captured some recently fed specimens inside mosquito curtains and Kirkpatrick (1925) states that in Egypt the adults enter houses and bite viciously after dark. We can find no records of experimental attempts to infect bred mosquitos of this species.

Wood (1915) dissected 37 *A. rhodesiensis*, caught in houses in Sierra Leone, and found 1 (2·7 per cent.) infected, whereas the sporozoite rates of *A. gambiae* and *A. funestus*, collected from the same places, were respectively 8·8 per cent. and 11 per cent.

Anopheles (Myzomyia) smithii Theobald (1905).

This species has, up to the present, only been recovered from one locality, Mount Aureol, near Freetown, Sierra Leone. According to Major Smith, R.A.M.C. [unpublished letter in the British Museum, quoted by Evans (1927)], females gorged with blood were taken on the barrack-room walls at Mount Aureol. There is no information as to the part it takes in the transmission of malaria.

Anopheles (Myzomyia) theileri Edwards (1912).

This mosquito has been found breeding in the neighbourhood of Freetown, by Blacklock and Evans (1926) and near Mabang, Sierra Leone, by Gordon (1929). No dissections have been made.

A study of these findings shows that in no species of anopheline recorded from Sierra Leone has it been shown that bred individuals develop sporozoites in the salivary glands after feeding on a suitable malaria carrier ; and with only one species, *A. funestus*, have convincing feeding experiments with wild mosquitos been carried out. The evidence obtained as a result of dissections of mosquitos found in houses is more adequate ; from this we are led to regard *A. gambiae* and *A. funestus* as probable transmitters, and *A. mauritianus*, *A. pharoensis* and *A. rhodesiensis* as possible transmitters.

It is obviously of the utmost importance to any Colony which is obliged to spend large sums of money annually in coping with its malaria problems, to have exact knowledge of the relative importance

of the existing anophelines. It was this absence of knowledge regarding the Sierra Leone species which encouraged us to undertake the observations which we record below.

As the work must, of necessity, be of an intermittent nature, we propose to publish our results as they are completed.

TECHNIQUE OF EXPERIMENTS

Throughout the experiments only bred mosquitos were used, the larvae of which were obtained from known breeding places around Freetown. With the exception of *A. smithii*, the adults were easily reared ; this latter species, as shown by Evans (1927), does not do well in captivity, but we overcame this difficulty by using a modification of Boyd's (1927) drip apparatus, which yielded a plentiful supply of adults. This apparatus consists of an upper reservoir containing a dilute emulsion of yeast in water, which discharges into a series of gauze protected jars in which the larvae are placed ; a small quantity of calcium carbonate is kept in each breeding jar, to conserve the alkalinity of the water. The details of the apparatus are sufficiently explained by the diagrams which illustrate this paper. The pupae were collected from the breeding jars daily and the imagines kept in cylindrical gauze-covered glasses, about four inches high and three inches in diameter. The jars were kept on trays covered with several layers of damp blotting paper to ensure sufficient humidity, and protected from ants by a water surround ; the whole apparatus being kept in a dark cupboard with a fairly constant temperature of about 25° C. In the intervals between feeding experiments, and before dissection, fresh raisins were placed on top of the mosquito netting and changed daily ; it was found necessary to cover these with a watch glass to prevent fruit flies breeding in them. Anophelines kept by this method remained alive for one or two months.

Feeding experiments were usually conducted after dark at about 6.30 in the evening. The patients were all native children receiving no quinine, and with a high proportion of gametocytes in their peripheral blood. In every case the number of gametocytes was checked by a thick film prepared from the patients' blood after the mosquitos had completed their feed. The following morning the

mosquitos were liberated inside a large cage, those containing blood being removed, and kept in separate jars, until ready for dissection, generally about twelve to fifteen days after the feed.
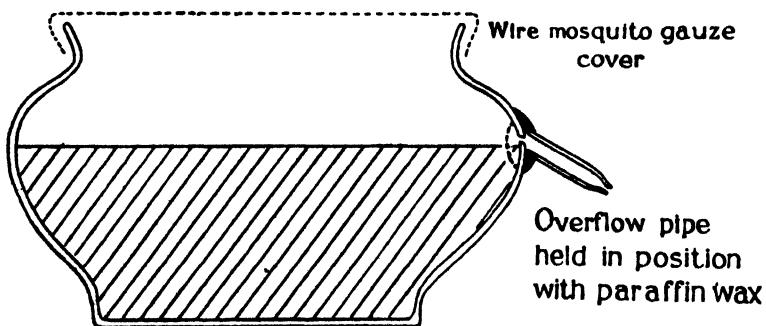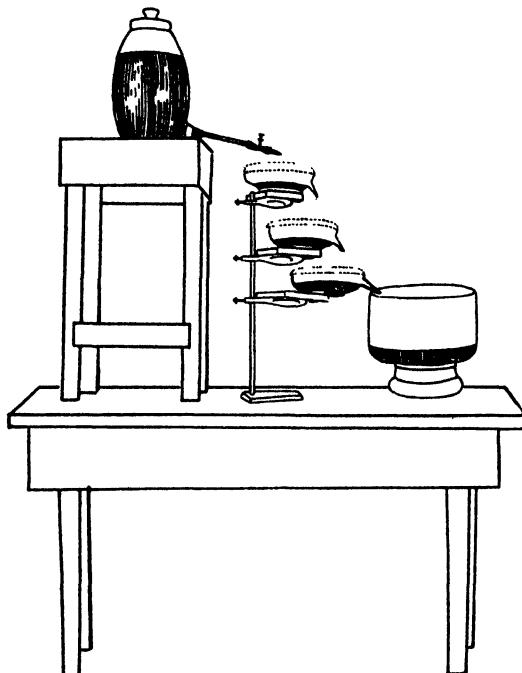
MODIFICATION OF BOYD'S DRIP APPARATUS FOR THE REARING OF ANOPHELINE LARVAE.

The glass dishes used are of the type employed for the exhibition of confectionery in shop windows. The hole for the overflow pipe is drilled with a diamond and provided with a fine wire gauze screen to prevent young larvae being washed away.

RESULTS OF EXPERIMENTS

A. gambiae.

Occurrence in Dwelling Places. This species is undoubtedly the commonest and the most widely distributed anopheline in Sierra Leone. In Freetown and, so far as we are aware, in other parts of the Colony, it is essentially a house haunting species and usually occurs in numbers far in excess of other anophelines, even when the larvae of the latter are found in greater numbers and closer to the habitation. Blacklock (1925) has drawn attention to this in comparing the numbers of *A. gambiae* and *A. nili* occurring in native huts at Daru and we, as we show later, have obtained similar results in the case of *A. gambiae* and *A. rhodesiensis.* Statements as to the preponderance of any species are, of course, only true of the actual period during which the observations are made ; a distinct seasonal variation being often observable. Thus, during a month's residence at Mabang, a village about forty miles from Freetown, we did not see a single specimen of *A. gambiae*, its place having been taken by large numbers of *A. mauritianus* and *A. squamosus.* On revisiting the same house six months later, not a single one of these species was observed, but large numbers of *A. gambiae* were captured on three successive nights.

Feeding Habits. In our experience *A. gambiae* feeds voraciously on man at all hours of day and night. Experiments with bred mosquitos showed that nearly 70 per cent. of those given an opportunity fed.

Natural Infections. We have already referred to the salivary gland infection rate as given by various writers ; the average of these results shows a rate of 7·1 per cent. ; our figure, derived from only 21 mosquitos caught in native huts at Mabang, was 9·5 per cent.

Experimental Infections. Of 21 *A. gambiae* fed on suitable native crescent carriers, three (14 per cent.) became infected in the salivary glands.

Conclusions. We have shown that *A. gambiae* fulfils all the conditions necessary to constitute it a vector of malignant tertian malaria. Its distribution and feeding habits in Sierra Leone undoubtedly render it the most important transmitter of this disease.

A. rhodesiensis.

Occurrence in Dwelling Houses. Gill (1916) considers this mosquito to be a domestic species and Wood (1915) found it in some numbers in dwelling places in Sierra Leone. During 1928 we searched a number of native houses in the immediate vicinity of two large breeding places near Freetown, and failed to recover a single specimen, though *A. gambiae* was relatively common. In one locality no *A. gambiae* breeding places were found in the neighbourhood of the houses, whereas *A. rhodesiensis* larvae were breeding in large numbers within 150 yards. During 1929 we had the opportunity of examining a number of native houses at the village of Aberdeen, near Freetown ; large numbers of *A. rhodesiensis* were found breeding in small rock pools about 10 feet above high tide level. A total of 117 anophelines were collected from houses situated close to these breeding places ; 5 of these proved to be *A. rhodesiensis*, the remaining 112 being *A. gambiae.*

Feeding Habits. We have some evidence from Gill (1916) that this species feeds on man in nature, he having found recently-fed specimens inside mosquito curtains. In captivity, however, we have found them most unwilling to feed on man, only about 10 per cent. of those given the opportunity drawing blood.

Natural Infections. We have done no dissections of wild *A. rhodesiensis* as we were unable to find them in sufficient numbers in houses. As stated before, however, Wood (1915) found one naturally infected specimen out of 37 dissected.

Experimental Infections. We have succeeded in feeding a total of 25 of this species, 18 on patients showing numerous crescents in their blood, and 7 on patients showing gametocytes of *Plasmodium malariae*. Of the 18 fed on cases of *P. falciparum*, 5 were dissected before the lapse of ten days, and 13 after ten days or more. One of the latter was found to have developed a fairly heavy infection of sporozoites in the salivary glands, the remaining 12 were negative. Six of the 7 fed on the patient with *P. malariae* were dissected after ten days, and 1 after six days only ; no developmental forms were seen in either the glands or gut.

Conclusions. Our experiments have proved that *A. rhodesiensis* can be successfully infected in the salivary glands as a result of feeding on a patient whose blood contains *P. falciparum* gametocytes, but when we consider their unwillingness to feed on man,

their comparative rarity in dwelling houses close to breeding places, the relatively low infection rate found by Wood (1915) and the small proportion of salivary gland infections which followed successful feeding on suitable carriers, we must come to the conclusion that *A. rhodesiensis* is not an important carrier of malaria in Sierra Leone.

A. smithii.

Occurrence in Dwelling Places. Major Smith, in a letter exhibited in the British Museum, states that he captured this species on the walls of the dormitories in the native Barracks on Mount Aureol. These Barracks have now been deserted for several years and we have failed to find adult mosquitos in the few scattered native houses still left inhabited in the district. At the time that we examined these native houses, numerous *A. smithii* breeding places existed in the vicinity and adults were captured resting on the rocks close to the breeding pools.

Feeding Habits. Evans (1927) found that bred females failed to feed on human blood, while under similar conditions 100 per cent. of bred *A. gambiae* engorged fully. In our experiments we also found it difficult to persuade captive *A. smithii* to feed on man. Of 63 which were given an opportunity to feed on native children, only 6 (9·5 per cent.) availed themselves of the invitation, while of 40 *A. gambiae* similarly applied to the skin of the same children, 27 fed. That they fed on man more readily in nature is suggested by the fact that the specimens collected by Major Smith, in the Mount Aureol dormitories, included gorged females.

Natural Infections. As already stated, we failed to obtain any specimens from houses.

Experimental Infections. Six *A. smithii* were successfully fed on natives, with sexual forms of *C. falciparum* in their blood, on dissection, thirteen days later, two (40 per cent.) were found infected with sporozoites in the salivary glands.

Conclusions. Our experimental proof of the susceptibility of *A. smithii* to infection with *P. falciparum*, together with Smith's record of the occurrence of fed females in native sleeping quarters, renders it likely that this species plays a small, but very local part in the transmission of malaria in the neighbourhood of Freetown, Sierra Leone.

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A COUNTING APPARATUS FOR USE WITH THE MICROSCOPE

BY

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PLATE I

The apparatus described is only intended for recording two sets of figures as, for example, the number of helminth ova occurring in a counted series of fields, or the proportion of one type of blood cell as compared with the sum total of other types ; it is, of course, not suitable for such work as complete differential leucocyte counts. The machine appears to possess two advantages over the usual method of writing down the figures as they occur : (1) It is more accurate and slightly more rapid ; (2) It saves the mental concentration required to carry a series of figures in the head, and any disturbance of attention which may arise during the count will not interfere with the accuracy of the result. This latter advantage appears to the writer to be most important. After the apparatus has been practised with for a short time, it will be found that the slight movement of the little finger of each hand, which registers each fresh figure, becomes entirely automatic and the mind of the observer is left free to consider the appearance of the objects under investigation. It is impossible to achieve such complete mental detachment if numbers have to be remembered and subsequently written down in special columns, after the examination of each microscopic field has been completed.

The apparatus consists of two revolution counters of the type employed in commercial works for attachment to revolving drums, etc. These counters, when purchased, are provided with five short spokes about 1 cm. long, and so arranged that a complete revolution moves the indicator five units and the movement required to bring

one spoke into the position previously occupied by the adjacent one
moves it one unit.

Before use, these spokes must be lengthened to about 4 cms. each.
This is most easily achieved by fixing a metal plate bearing the
five 4 cm. spokes over the old plate and short spokes. The counters
are not provided with any stops, so that it is necessary to
fit them with watch spring checks so arranged (see diagram) that each
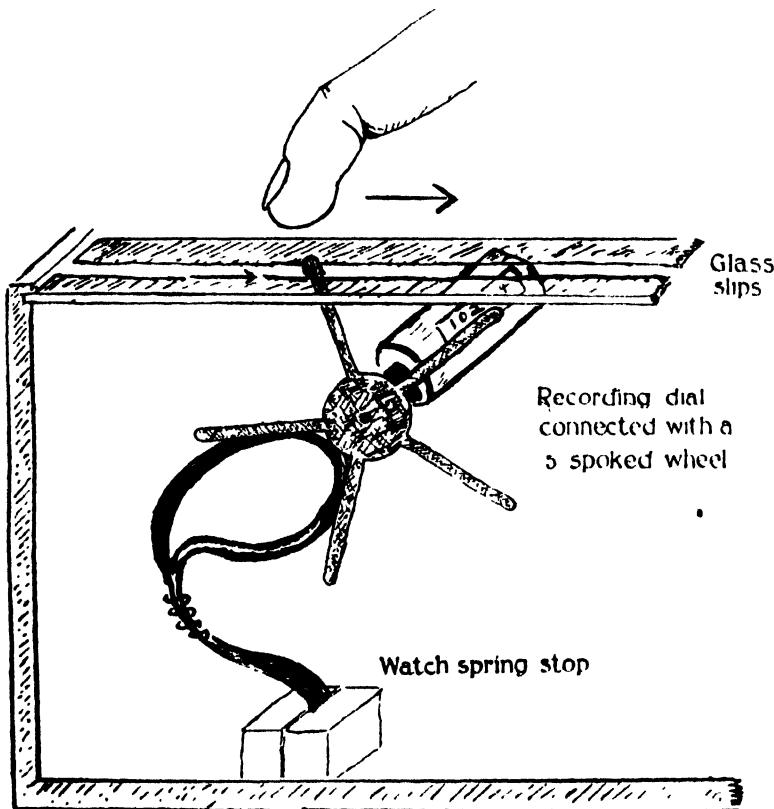
one-fifth of a complete revolution brings a fresh spoke and,
consequently, a new number on the recording dial, into position.
It is important that the watch spring checks should be so adjusted
that they will record this movement with an audible click. The
revolution counters are mounted inside two wooden boxes about
11 cms. high and about 15 cms. apart ; the most convenient height

and distance apart varying slightly with the type of microscope in use and the individual requirements of the user. The counters and their stops should be mounted on wooden blocks at such a height inside the boxes that the uppermost spoke of the apparatus will project 1 cm. above the top of the box. The revolution of the spokes takes place between two slips of glass let into the top of the box. These glass slips form a rest for the little fingers of each hand and allow of the recording dial being read without exposing the machinery to dust.

The writer is indebted to Mr. A. E. Goodrich, of the Liverpool School of Tropical Medicine, for making the model illustrated.

EXPLANATION OF PLATE I

Λ counting apparatus for use with the microscope.

    Top :   Apparatus in use.
    Bottom : Apparatus as seen from above.
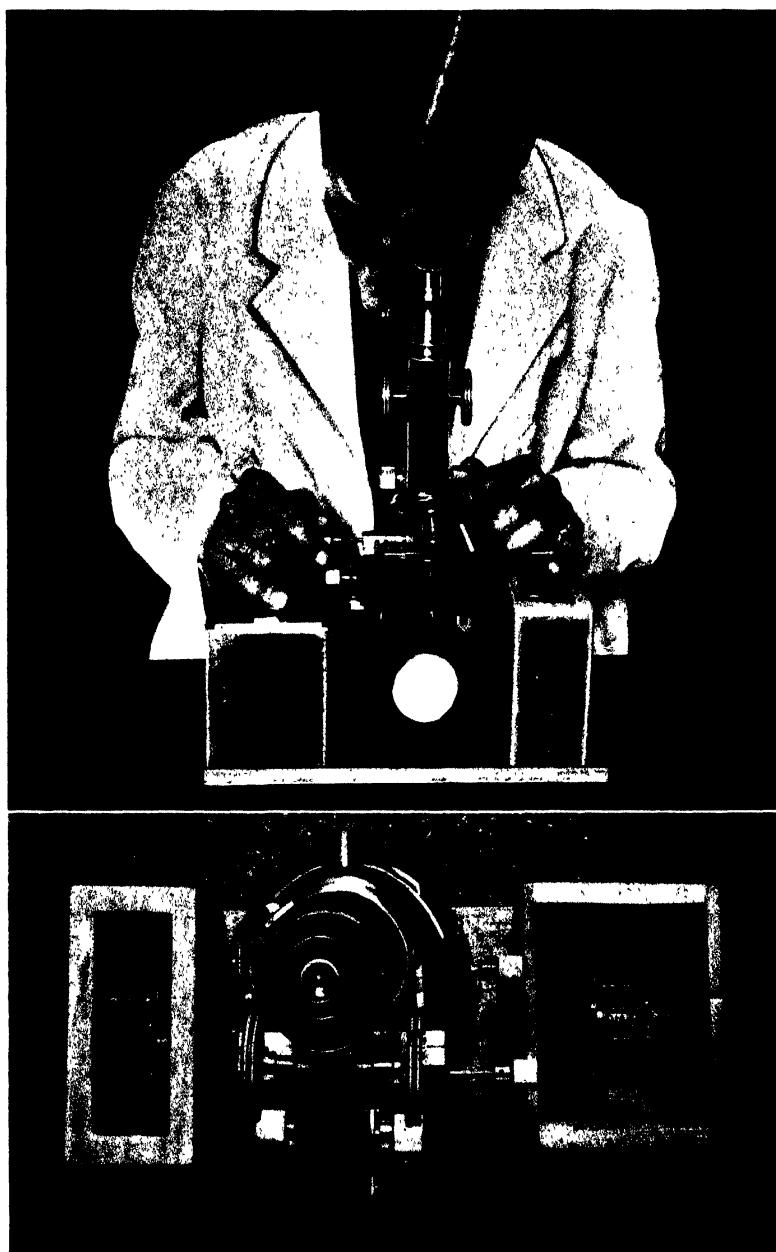
NOTES ON *CATHAEMASIA HIANS* (Rudolphi) FROM THE MOUTH OF *CICONIA NIGRA*

BY

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PLATE II

The worm was obtained from the mouth of a *Ciconia nigra* which is being kept at Tennoji Zoological Gardens in Osaka. From the external characters and internal structures we believe that it is identical with *Cathaemasia hians* (Rudolphi, 1809).

There are several descriptions of this species, but some of them are fragmentary and they differ one from another in various respects. We intend, therefore, to describe briefly our observations on this parasite.

EXTERNAL CHARACTERS.

The living worm is pink to cream in colour and in physiological salt-solution it exhibits rapid movements in expansion and contraction of its body ; the anterior part of the body is especially mobile. It is difficult to measure its size accurately during life. The following measurements of various parts of the body were obtained from specimens preserved in 10 per cent. formalin.

1. *Form and size.* The body is elongated, oval, flattened dorso-ventrally, and from the ventral sucker it becomes gradually narrower towards the anterior end, the posterior extremity being broadly rounded. The length varies from 9·0 mm. to 13·75 mm., and the breadth from 3·42 mm. to 4·69 mm. The thickness is usually considerable.

2. *Cuticle and spines.* The whole body is covered by the cuticle, which is more or less wrinkled, its thickness being greater in the median portions of both dorsal and ventral surfaces than in the lateral margins. There is no conspicuous difference in thickness between the dorsal and ventral cuticle which is about 0·022 mm. to 0·048 mm. thick. As in other species of this genus, the worm is

provided with spines or scales on the ventral surface except over a small area at the cephalic and caudal extremities, and a small portion around the ventral sucker. Their arrangement and variation in size are very regular ; they are small and closely set in the anterior part of the body and gradually increase in size and are sparsely distributed in the caudal region ; they are rectangular, square or oblong in shape. The spines in the anterior region are short but posteriorly they increase in length more quickly than in breadth ; also they vary in form from oblong to square. The posterior oblong spines have usually a narrow or more or less pointed end ; the median spines are a little larger than those of the lateral margins. The measurements of the spines in various parts of the body of the larger specimens are as follows :—

0·045 mm. to 0·048 mm. broad and 0·032 mm. to 0·045 mm. long, just behind the oral sucker ; 0·064 mm. to 0·080 mm. broad, and 0·08 mm. to 0·097 mm. long posteriorly to the ventral sucker ; 0·097 mm. to 0·112 mm. long between the two testes. On the surface of the spines there are longitudinal striations, the number of which increases posteriorly.

TABLE I

Measurement of scales of large specimen, in mm.

		Breadth	Length	Number of striations
Oral sucker	Just behind	mm. 0·045–0·048	mm. 0·032–0·048	...
	Lateral margin	0·032	0·032–0·040	...
At point of origin of intestinal caeca...	Median	0·045–0·064	0·080	1–2
	Lateral margin	0·064	0·074	3–4
Ventral sucker	Lateral	0·064	0·080	3–4
Ventral sucker	Just behind	0·064–0·080	0·080–0·097	3–4–5
	Lateral margin	0·064	0·064–0·080	3–4
Ovary	Median	0·097	0·129–0·145	5–6–7
	Lateral margin	0·064–0·080	0·097–0·112	...
Between two testes	Median	0·097	0·129–0·145	Numerous
	Lateral margin	0·064–0·080	0·097–0·112	Numerous
Behind posterior testis	...	0·06–0·064	0·080	Numerous

TABLE II

Measurement of scales of medium specimen in mm.

		Breadth	Length	Number of striations
Oral sucker	Just behind	mm. 0·032	mm. 0 026	...
	Lateral margin	0·016	0·026	...
At point of origin of intestinal caeca...	Median	0·032	0·064	2–3
	Lateral margin	0·032	0·048	2–3
Ventral sucker	Lateral	0·042	0·048	...
Ventral sucker	Just behind	0·048	0·048	...
	Lateral margin	0·042	0 048	...
Ovary	Median	0·048–0·051	0·064–0·074	...
	Lateral margin	0·048	0·048	...
Between two testes	Median	0·064	0·064–0·071	...
Behind posterior testis	..·,	0·064	0·064	...

3. *Suckers.* The oral and ventral suckers are circular, or almost so ; the former being subterminal and smaller than the latter. The oral sucker is about 0·65 mm. to 0·93 mm. by 0·40 mm. to 0·84 mm., while the ventral sucker is 0·97 mm. to 1·97 mm. by 0·94 mm. to 1·53 mm. in diameter. The distance between the two suckers is 2·25 mm. to 3·19 mm., the ventral sucker being situated at about one-third the distance from the anterior end of the body.

INTERNAL STRUCTURE.

1. *Musculature.* The musculature consists of outer circular, middle longitudinal and inner diagonal muscles. Besides the above the dorso-ventral muscles are well developed. There is no obvious difference of development between the circular muscles on the dorsal and ventral aspects ; the thickness varies from 0·016 mm. to 0·036 mm. In general, the circular muscles near the lateral margins are somewhat thinner than in the median portion. The well-developed genital organs appear to hinder the development of the circular muscles. The longitudinal muscles are more strongly developed on the ventral side than on the dorsal. Their thickness ventrally is 0·064 mm. to 0·161 mm., while dorsally it is only 0·032 mm. to

0·046 mm. The longitudinal muscles also decrease in thickness laterally ; the diagonal are as well developed as the circular muscles. From such a strong development of the musculature results the actively mobile character of this worm, a feature which is most necessary in order to adapt it to its habitat.
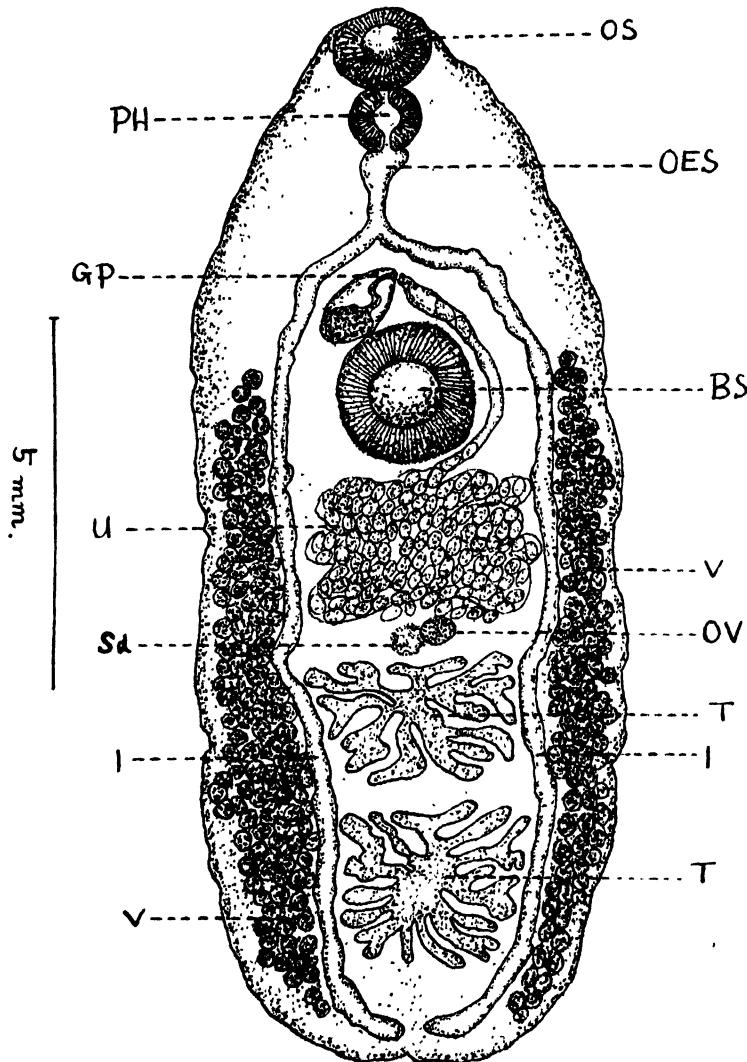
Cathaemasia hians (Rud.)

BS—Ventral sucker ; *GP*—genital opening ; *I*—intestinal caeca , *OES*—oesophagus ; *OS*—oral sucker ; *OV*—ovary ; *PH*—pharynx ; *Sd*—shell gland ; *T*—testis ; *U*—uterus ; *V*—vitellaria.

TABLE III

Measurement of cuticle and muscle layers in mm.

		Ring muscle	Longitudinal muscle	Diagonal muscle	Cuticle	Body thickness
At point of origin of intestinal caeca	Dorsal	mm. 0·032	mm. 0·042–0·048	mm. 0 022	mm. 0·032	mm. 0·84
	Ventral	0·032	0·064–0·145	0·048	0·016–0·022	
Ventral sucker ...	Dorsal	0·032	0·040	0·040	0·020–0·032	1·0
	Ventral	0·032–0·038	0·161	0·048	0·048	
Ovary	Dorsal	0·016–0·025	0·032–0·048	...	0·025	1·1
	Ventral	0·032	0·161	0·016–0·025	0·032	
Anterior testis ...	Dorsal	0·032–0·040	0·048	...	0·022–0·032	1·0
	Ventral	0·048	0·097–0·129	0·032	0·016–0·032	

2. *The digestive organs.* From the oral sucker there arises a short prepharynx ; the pharynx, measuring from 0·45 mm. by 0·54 mm. to 0·56 mm. by 0·65 mm., is succeeded by a fairly long oesophagus which divides at an acute angle to form the intestinal caeca at a point about 0·51 mm. to 0·74 mm. posterior to the pharynx. The intestinal caeca are long, straight, unbranched tubes, running approximately parallel to the lateral margin of the body. The point of division into the intestinal caeca is 1·45 mm. to 3·55 mm. from the cephalic end of the body, and lies about midway between the two suckers. The intestinal caeca come nearer together as they approach the posterior end of the body.

3. *The female organs.* The ovary lies in the median line in front of the anterior testis, and is covered by a loop of the uterus. It is nearly oval in shape, measuring about 0·23 mm. to 0·37 mm., by 0·14 mm. to 0·25 mm. in diameter. The shell gland is about the same size and lies in contact with the ovary. The vitellaria lie externally to the intestinal caeca, one on each side, and arise anteriorly a little in front of the ventral sucker ; they extend to the posterior extremity. A definite receptaculum seminis appears to be absent ; a Laurer's canal is present. The uterus occupies the inter-caecal space between the ovary and the genital pore ; behind the ventral sucker it is thrown into several transverse coils. It then pursues an almost straight course and becomes the metraterm, which is a

straight canal opening into the genital sinus. The latter is about equidistant from the ventral sucker and the bifurcation of the intestinal caeca. The eggs are rather large in size, light yellow in colour, oval in shape, one end being rounded and the other a little pointed. The eggs have a small knob-like projection at the anti-opercular pole ; they measure 0·072 mm. to 0·083 mm. in length, 0·041 mm. to 0·055 mm. in breadth, the average size being 0·075 mm. by 0·045 mm.

4. *The male organs.* The testes, two in number, lie in the median line, at the anterior part of the posterior half of the body, one behind the other. Both testes are deeply lobed, having branches which are disposed radially. The form of the testes appears to be the most important character by which the species of this genus are identified, but opinions vary on this subject. We shall deal with this in detail later on in the present paper. From each testis a vas deferens arises which pursues an almost straight course and unites with the other one to enter into the cirrus pouch which lies in front of the ventral sucker. Within the cirrus pouch, the vas deferens dilates into a seminal vesicle which is followed by the pas prostatica and the ductus ejaculatorius in succession. The latter runs straight to the male opening situated near that of the female.

TABLE IV

Measurements of, and various parts of, some specimens in mm.

Specimen	No. 1	No. 2	No. 3	No. 4	Average
	mm.	mm.	mm.	mm.	mm.
Body—Length ...	13·85	10·14	9·15	9·00	10·56
Breadth ...	4·69	3·65	3·55	3·42	3·82
Oral sucker	0·93×0·84	0·69×0·42	0·65×0·40	0·64×0·40	0·73×0·51
Ventral sucker ...	1·59×1·53	1·12×1·08	0·99×0·97	0·97×0·94	1·17×1·13
Distance of two suckers	3·19	2·41	2·34	2·25	2·55
Pharynx	0·65×0·56	0·59×0·52	0·56×0·50	0·54×0·49	0·58×0·52
Oesophagus-length ...	0·74	0·56	0·56	0·50	0·59
Ovary	0·37×0·25	0·26×0·17	0·26×0·16	0·23×0·14	0·28×0·18
Distance of branching points from anterior end	3·55	1·75	1·68	1·46	2·11
Cirrus pouch ...	1·05×0·75	0·68×0·42	0·67×0·41	0·62×0·40	0·76×0·49
Egg	0·083×0·055	0·071×0·043	0·073×0·041	0·072×0·041	0·074×0·045

AFFINITIES. Hitherto four species of this genus have been described : *Cathaemasia hians* (Rudolphi, 1809) ; *C. fodicans* Braun, 1901 ; *C. spectabilis* Odhner, 1926, and *C. famelica* Odhner, 1926 ; of these, *C. hians* has been described by several writers, the others only once by their authors. Braun distinguished *C. fodicans* from *C. hians* chiefly by the deep branching of the testes and the smallness of the eggs. Recently, Odhner published his opinion that *C. fodicans* is not strictly separable from *C. hians*, because the testes of *C. hians* are also deeply branched, not as in the figure given by Mühling. Braun also stated that he could not find scales on his species. It is probable, however, that the worm was originally provided with scales which had fallen off during preservation. Agreeing with Odhner's opinion, we believe that *C. fodicans* is probably identical with *C. hians* (Rudolphi).

Fig 1
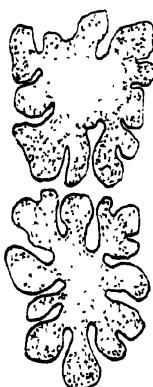
Fig 2

Fig. 3

C. fodicans Brn.
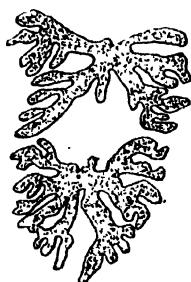
C. spectabilis Odh.

C. famelica Odh.

Fig. 4
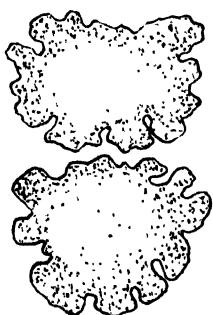
Fig. 5
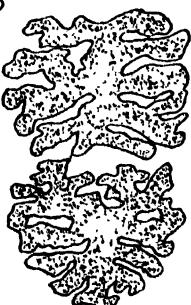
Fig. 6

C. hians. Rud.
(Mühling)
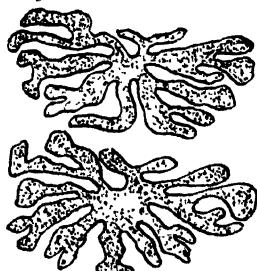
C. hians Rud.
(Odhner)

C. hians Rud.
(Yoshida et Toyoda)

The present parasite is obviously different from *C. spectabilis* in many respects, especially in size of body, distribution of the scales, branching manner of the testes and the size of the eggs. From *C. famelica* the parasite is distinguished by the form of the testes and by the shape and size of the ovarium, characters which Odhner utilised for separating his species from *C. hians*. In various characters the present worm agrees with *C. hians*. The branching of the testes is a little different from the figure given by Mühling, but quite the same as Odhner's figure of the testes.

THE LIVING WORM. This species is reported to have been found in the oesophagus of the host, but we found it in the mouth cavity only. It is obvious that the parasite lives in the oesophagus and sometimes proceeds to the mouth cavity. We made an interesting observation on the behaviour of the living worm. We noticed that when the host appeared to cough and to become hoarse, we found worms in the mouth cavity if we quickly examined it. But if we were not able to open the mouth very quickly, all the worms disappeared, apparently down the oesophagus. We made this observation very often in the Zoological Gardens, as well as in our laboratory. Mr. Hayashi, the chief of the Zoological Gardens, was so kind as to offer us a stork for our personal observation in our laboratory. The chief experts and gardeners informed us that at the time when the host in question, *Ciconia nigra*, was sent from its native land, Africa, it often emitted a number of worms from its mouth, but as they did not know what they were, they trampled them to death. At that time the bird seemed to be quite sick, but no one recognised the cause. The symptoms gradually disappeared as the number of worms decreased. Recently the bird has been in a good condition of health, and the parasite is rarely found in the mouth, so that it is very difficult to obtain specimens of the worm.

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PLATE II

EXPLANATION OF PLATE II

Cathaemasia hians (Rud.)
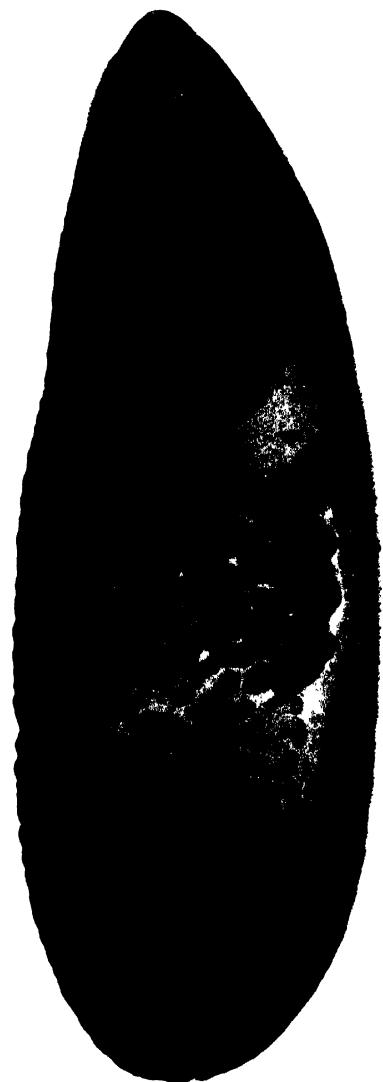
ON THE SUSCEPTIBILITY OF THE TWO SEXES OF *G. PALPALIS* TO INFECTION WITH *T. GAMBIENSE* AND *T. RHODESIENSE*

BY

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In a previous paper (1928b) the effect of trypanosome infections on the longevity of *G. palpalis* hatched at the Entebbe Laboratory was discussed and the conclusion reached that the developing flagellates did no harm to the fly. The experimental records of the transmission experiments carried out at this Laboratory during the three years from August, 1926, to July, 1929, have now been examined to see whether they afford any indication of a difference in susceptibility to infection between male and female *G. palpalis*. The essential organic differences between the two sexes are accompanied by certain minor peculiarities. The females are, on the average, larger than the males, and feed generally more voraciously. Partly, no doubt, in consequence of this propensity, traces of blood are often discernible for a longer period in female than in male flies killed forty-eight hours after their last meal ; but this phenomenon is also associated with and doubtless partly explained by the coincidence of pregnancy.

For the purposes of this review, all experiments that contained at least one infected fly were selected and the figures noted. Negative experiments were ignored. The total number of laboratory-bred *G. palpalis* coming thus under review was 24,509, of which 12,737 were males and 11,772 were females. Of this total, 1,237 (5 per cent.) contained flagellates—developmental forms of either *T. gambiense* and *T. rhodesiense*. Of these infected flies, 581 were males and 656 females ; i.e., 4·5 per cent. and 5·5 per cent., respectively.

There remained the possibility that there might be a difference between the two sexes in relation to the final stage of the developmental cycle in the fly, namely, the invasion of the glands.

Examined from this aspect the figures yielded the following information. Of the 12,737 males exposed to infection, 178 (1·3 per cent.) had flagellates established in their salivary glands ; of the 11,772 females, 183 (1·5 per cent.) were similarly infected. That is to say, of the ' positive ' male flies, 30·6 per cent. showed gland infections ; of the ' positive ' females, 27·8 per cent.

The strains of the trypanosomes under experiment during these three years differed considerably in their transmissibility. Some were at the stage where the power to invade the glands had disappeared, the flagellates still retaining the power of developing in the gut of the fly. Indeed, many of the experiments included in this review were performed with strains of very low transmissibility ; strains, in fact, that had, in the course of prolonged upkeep in a single individual host, become non-transmissible and all but non-infective to tsetse (Duke, 1927). Thus the above percentages have no general value as an indication of the susceptibility of G. *palpalis* to infection with T. *gambiense* and T. *rhodesiense*. They are useful merely for the special purpose for which we have employed them, i.e., the comparison of the behaviour of the two sexes under identical conditions.

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A NEW ANAPORRHUTINE TREMATODE GENUS AND SPECIES *NAGMIA YORKEI*, WITH A REVIEW OF THE CLASSIFICATION OF THE SUB-FAMILY

BY

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Nagmia yorkei n.gen., n.sp.

Five trematode worms were found in a bottle which also contained a number of cestodes. The bottle was labelled, ' Worms from the rectum of *Trygon* sp., Pearl Banks, Ceylon.' Whilst there can be no question that the cestodes were found in the spiral valve, it is important to note that trematodes allied to the species to be described usually occur in the body cavity around the liver and in the pericardium of certain fishes.

The parasites are fairly fleshy, slightly concave ventrally and convex dorsally. The anterior extremity is produced into a conical projection, the rest of the worm being circular. They measure from 1·6 cm. to 1·7 cm. in length, and from 1·2 cm. to 1·6 cm. in breadth. The body is smooth and does not bear either scales or spines. The oral sucker is situated ventrally at the anterior extremity. The ventral sucker, which is not elevated from the ventral surface, has an average diameter of about 2·7 mm. The distance between the two suckers is about 3·7 mm. The genital openings are placed in the mid-longitudinal axis of the body between the inner borders of the intestinal caeca and about 500μ posterior to their bifurcation. They are situated about 1·7 mm. behind the posterior border of the oral sucker and about 2 mm. in front of the anterior border of the ventral sucker. Slightly anterior and lateral to the female genital opening, in one specimen only, a third small sucker was noted. This has a thick muscular wall, with radially arranged fibres. The diameter of the outer contour of this wall is about 200μ.

Digestive System. The oral sucker measures about 1·3 mm. antero-posteriorly, and 1·9 mm. transversely. It is succeeded by a strongly developed muscular pharynx which opens anteriorly in the floor of the oral sucker. The pharynx is almost spherical and more than its anterior half is situated under the posterior lip of the
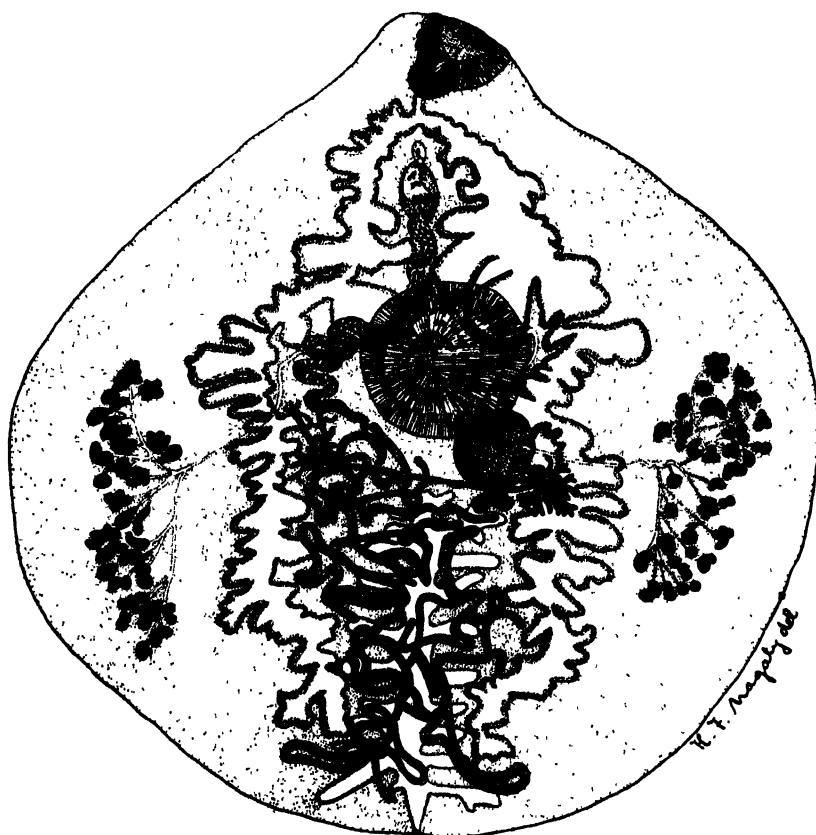
FIG. 1. *Nagmia yorkei*, n.g., n.sp. Ventral view.

(Magnification approximately 7 times.)

oral sucker. It measures about 720μ antero-posteriorly, and about 880μ transversely. The oesophagus is very short, measuring about 200μ in length ; that part immediately behind the pharynx is narrow, but it gradually widens posteriorly towards the intestinal caeca. It measures at its narrow anterior end about 200μ, and at

the wide posterior extremity about 300μ in breadth. The intestinal caeca are situated midway between the mid-longitudinal axis of the worm and its lateral borders; they are so sacculated as to appear branched, the sacculations being sometimes as thick, or even thicker, than the main caeca themselves. The contents of the caeca and their branches stain deeply and there is always a clear space between the contents and the walls, as observed by Looss and Ofenheim in other distomes. The breadth of the intestinal caeca ranges from 380μ to 1·3 mm. The longest sacculation measured 1·5 mm., and the shortest 190μ; they are globular or oval and usually open into the main caeca by narrow mouths. Their breadth varies from 1·7 mm. to 190μ. The intestinal caeca terminate at a distance varying from 1·3 mm. to 2 mm. from the posterior border of the parasite.

Male Genitalia. There are two lobed testes. Each occupies an elongated area, this being roughly the middle third of the length of the worm external to each intestinal caecum. They are situated midway between the outer borders of the intestinal caeca and the lateral margins of the parasite, and in some cases they are slightly nearer the former than the latter. Each testis consists of from twenty-nine to thirty-five lobes, which are not arranged in a special manner. These lobes are irregular in shape and measure from about 280μ by 300μ to 760μ by 630μ. Vasa efferentia arise from these lobes and converge towards the inner side of the area occupied by the testes where they all meet in the centre of the inner border of this area and form the main vas deferens which has an average diameter of 50μ. It crosses the intestinal caecum from the outer to the inner side, ventrally, and as soon as it gains this side it makes its way towards the anterior extremity in close proximity to the inner wall of the intestinal caecum of its own side. The vasa deferentia of the two sides meet at a distance of about 730μ in front of the anterior border of the ventral sucker, between the two intestinal caeca, and in the middle line. The duct formed by the union of the two vasa deferentia runs for about 320μ before it dilates into a vesicula seminalis; no cirrus pouch is present. The former organ is sac-shaped and is situated between the two intestinal caeca opening slightly posteriorly to, or at the same level as, the female genital opening. The shape of the sac differed in the specimens examined.

The vesicula seminalis and the main vas deferens connected to it are situated dorsally to the distal part of the uterus.

Female Genitalia. The ovary and receptaculum seminis lie between the intestinal caeca towards the right or the left side of the median line at about the same distance from the anterior as from the posterior extremities of the worm. The ovary is an elongated bilobed or trilobed organ lying immediately posterior to, and in close contact with, the receptaculum seminis; it measures about 935μ by 355μ.
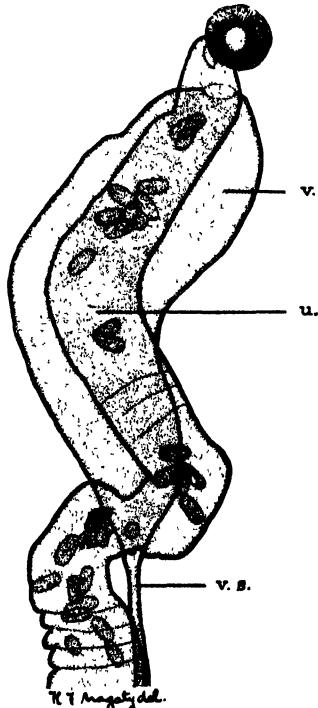
Fig. 2. Terminal part of the genital ducts of *Nagmia yorkei*, n.g., n.sp. Ventral view. (× 54.)

The receptaculum seminis is a spherical organ very large in proportion to the size of the parasite and situated anteriorly to the ovary; its outer border is in contact with the inner wall of the intestinal caecum of its side, and its average diameter is about 2 mm.

The vitellaria consist of two glands connected together by a transverse duct. The glands and their ducts lie in the mid-

transverse axis of the worm or slightly behind it. Each gland lies ventrally and internally to the intestinal caecum of its side ; that on the side on which the receptaculum seminis lies is posterior and lateral to this organ. Each gland is composed of many tubules, numbering from about twenty to thirty ; from four to ten of these unite together and form a main stem. Three or four such stems are formed in all and these unite together. The tubules lie dorsoventrally and measure from 80μ to 130μ in breadth ; the main stems measure about 160μ in breadth. The transverse duct connecting the two vitelline glands passes between the ovary and the receptaculum seminis. The duct is not of uniform diameter and measures from 50μ to 160μ in diameter.

The uterus, which was full of eggs, consists of a coiled tube with a more or less uniform diameter of about 190μ. It is very coiled in the space which it occupies behind the vitelline duct and the inner walls of the intestinal caeca. Anteriorly to the vitelline duct the distal part of the uterus consists of a coiled tube which is of a slightly wider diameter than the rest of the uterus. The coiling of the distal part of the uterus nearest to the vitelline duct consists of a more or less regularly zig-zagged tube. It passes either to the right or left side of the ventral sucker.

FIG. 3. Eggs from uterus of *Nagmia yorkei*, n.g., n.sp. ($\times$ 139.)

The eggs are elongated and rounded at both ends. They are operculated and in many instances the lid was found open inside the uterus, probably as a result of pressure. They possess thin, clear shells and measure 96μ by 48μ.

The Excretory Vesicle. This extends from the level of the ovary to the posterior margin of the parasite, and opens by an excretory pore on the ventral surface very near its posterior margin. The vesicle has a main stem extending along the longitudinal axis of the worm, with lateral branches along its length, which extend laterally as far as the inner walls of the intestinal caeca.

NOTE.—For diagnosis of the genus *Nagmia*, see page 107.

In another bottle there were about forty worms which, on examination, proved to be specimens of *Staphylorchis largum* (Lühe, 1906), Baylis 1927. The bottle was labelled as follows : ' Parasites from *Ginglymostoma concolor* Periya Paar, Pearl Banks, Ceylon.' From which part of the fish they were collected is not stated.

The single worm of this species upon which Lühe based his description was from the body cavity of *Rhinoptera javanica* Kalpitiya. Kalpitiya is very near Periya Paar and is therefore the same locality.

Nothing much can be added to Lühe's description and figures, but the opportunity is here taken to supplement his account from the large number of specimens available.

The worms are very transparent and membranous, and measure from 1·1 cm. to 2·3 cm. in length, by from 9 mm. to 20 mm. in breadth; the posterior border bears an indentation which resembles an inverted **V**. The ventral sucker is large in proportion to the size of the worm, shallow, much elevated from the ventral surface, and for this reason very conspicuous and characteristic. The intestinal caeca are either straight or slightly sinuous. The opening of the male and female genital ducts is anterior to the bifurcation of the intestinal caeca, immediately behind the posterior margin of the muscular pharynx. The lobes of the testes are spherical and number from nine to twenty-one on each side, occupying only a small area. The ovary is spherical and may be to the right, to the left or posterior to the receptaculum seminis ; the excretory vesicle is **Y**-shaped. The receptaculum seminis is spherical, larger than the ovary, and situated dorsally to the ventral sucker either partially or completely anterior to its posterior border. The uterus was noticed, in these specimens, to occupy a narrow area in the middle line antero-posteriorly and is dorsal to the excretory vesicle ; it does not occupy all the area between the intestinal caeca as figured by Lühe. Its terminal part passes either to the right or to the left of the ventral sucker.

Discussion. Looss (1901) divided the family Gorgoderidae into two sub-families, viz., Gorgoderinae and Anaporrhutinae. He further included in the latter sub-family the three genera, *Anaporrhutum*, *Probolitrema* and *Plesiochorus*.

Since then several new genera have been described and added

to this sub-family, which now contains the following : *Anaporrhutum* Ofenheim, 1900 ; *Probolitrema* Looss, 1901 ; *Plesiochorus* Looss, 1901 ; *Petalodistomum* Johnston, 1912 ; *Staphylorchis* Travassos, 1920. Travassos erects the new genus *Dendrorchis* and classifies it as one of the genera belonging to the sub-family Anaporrhutinae. The writer has found that this genus is a synonym to the genus *Phyllodistomum* Braun, 1899, which belongs to the sub-family Gorgoderinae.

I have emended the diagnosis of the genus *Petalodistomum* Johnston, 1912, after Travassos had erected the new genus *Staphylorchis* to accommodate *Petalodistomum cymatodes* ; the other genera have been also emended for the sake of uniformity.

Diagnosis of the sub-family ANAPORRHUTINAE Looss, 1921.

Medium to large Gorgoderidae with the posterior part of the body distinctly broad. Muscular pharynx present and usually connected with a short oesophagus. The terminal part of the genital tract is strongly developed. The vesicula seminalis is long and coiled. The ejaculatory duct and the metraterm (terminal part of the uterus) is elongated. Female genital organs without Laurer's canal, but with a strongly developed receptaculum seminis. Testes may be internal or external to the intestinal caeca. Vitelline glands apart from each other.

Diagnosis of the genus *Anaporrhutum* v. Ofenheim, 1900.

Large Anaporrhutinae with definitely broadened posterior part of the body. A strong muscular pharynx present ; intestinal caeca not branched. The testes are divided into a large number of spherical bodies which are partly internal and partly external to the intestinal caeca. Vitelline glands are ventral and internal to the intestinal caeca. A large receptaculum seminis present. In the pericardium and body cavity of Elasmobranch fishes.

Type-species :—*Anaporrhutum albidium* v. Ofenheim, 1900.

Ofenheim (1900) also included in this genus *Anaporrhutum ricchiardii* ; Looss (1901) made a new genus to accommodate this species on account of the fact that it differed from *Anaporrhutum albidium* in that the vitelline glands and the testes are completely outside the intestinal caeca, a difference of generic value.

Table showing the main differences in the genera of the sub-family ANAPORRHUTINÆ.

	Anaporrhutum	Probolitrema	Plesiochorus	Petalodistomum	Staphylorchis	Nagmia
Muscular pharynx ...	Present.	Present.	Present.	Present.	Present.	Present.
Oesophagus	Absent.	Very short.	Short.	Short.	Short.	Short.
Intestinal caeca ...	Unbranched.	Unbranched.	Unbranched.	Branched.	Sinuous but unbranched.	Branched.
Shape of testes ...	Divided into a large number of small, smooth, spherical bodies.	Divided into a large number of irregularly-shaped bodies.	Simple but deeply lobed.	Deeply lobed and divided into several distinct large pieces.	Divided into a large number of small spherical bodies.	Divided into a large number of irregularly-shaped bodies.
Relation of testes to intestinal caeca.	Partly internal and partly external.	Definitely external.	Partly internal and partly external.	Definitely external.	Definitely external.	Definitely external.
Position of testes ...	In the middle third of the body.	In the middle third of the body.	In the posterior half of the body.	In the posterior half of the body.	In the middle and posterior thirds of the body.	In the middle third of the body.
Receptaculum seminis.	Present.	Present.	Present.	Present.	Present.	Present.
Shape of vitelline glands.	Finely dendritic.	Finely dendritic.	Coarsely dendritic.	Composed of small spherical follicles.	Small follicles. Some spherical, some elongated.	Corasely dendritic.
Relation of vitelline glands to intestinal caeca.	Ventral and internal.	Definitely external.	Partly internal and partly external.	Internal.	Definitely internal.	Ventral and internal.
Position of vitelline glands.	In the anterior half of body.	In the anterior half of body.	In the anterior half of body.	Near mid transverse line.	In the anterior half of the body.	Near mid transverse line.
Shape of excretory vesicle.	T-shaped.	Y-shaped.	Y-shaped.	Branched.	Y-shaped.	Branched.
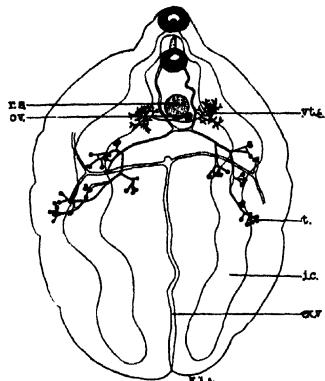
(a) Scheme of the genus *Anaporrhutum*
Size of the type species is from 7·8 mm. × 4·8 mm.
to 3·1 cm. × 1·2 cms.
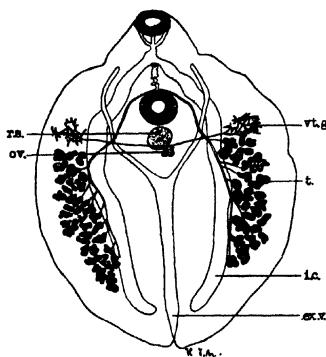
(b) Scheme of the genus *Probolitrema*.
Size of the type species is from 6 mm. × 4·5 mm.
to 1·9 cm × 1·3 cm.
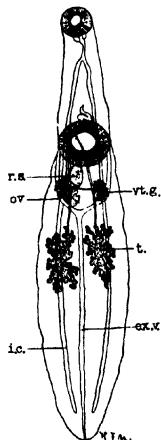
(c) Scheme of the genus *Plesiochorus*.
Size of the type species is from 2 mm. long to
1·2 cm. × 2·4 mm.
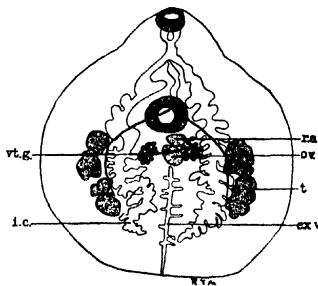
(d) Scheme of the genus *Petalodistomum*.
Size of the type species is from 3·3 × 3 mm. to
3·76 × 3·5 mm.
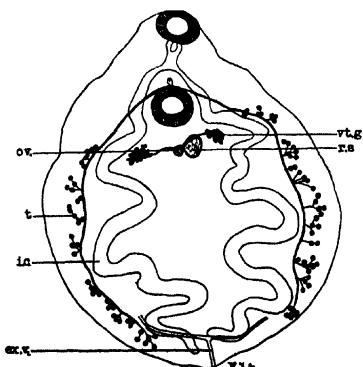
(e) Scheme of the genus *Staphylorchis*.
Size of the type species is 10·5 mm. × 8 mm.
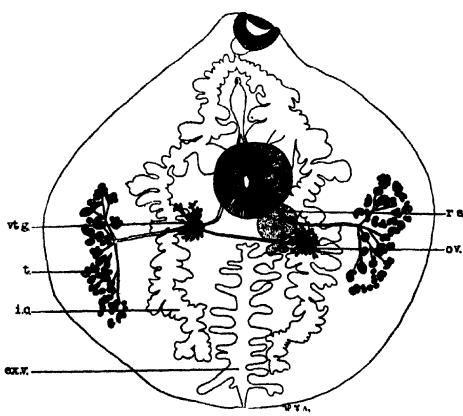
(f) Scheme of the genus *Nagmia*. n.gen.
Size of the type species is from 1·6 × 1·2 cm. × 1·7 × 1·6 cm.

Diagnosis of the genus *Probolitrema* Looss, 1901.

Large Anaporrhutinae with definitely broadened posterior part. A strong muscular pharynx present ; intestinal caeca not branched. The testes are divided into a large number of irregularly shaped bodies and together with the vitelline glands are definitely external to the intestinal caeca. A large receptaculum seminis present. In the body cavity of Elasmobranch fishes.

Type-species :—*Probolitrema ricchiardii* (Lopez, 1888), Looss, 1901.

Diagnosis of the genus *Plesiochorus* Looss, 1902.

Middle-sized Anaporrhutinae with a fairly broad posterior end of the body. A strong muscular pharynx present ; intestinal caeca not branched. Testes simple but deeply lobed and like the vitelline glands ventral and partly internal and partly external to the intestinal caeca. A large receptaculum seminis present. In the urinary bladder of the marine Chelonia.

Type-species :—*Plesiochorus cymbiformis* (Rud., 1819), Looss, 1901.

Diagnosis of the genus *Petalodistomum* Johnston, 1912.

Middle-sized Anaporrhutinae with the posterior part of the body very broad, almost circular and plate-like. A strong muscular pharynx present ; intestinal caeca branched. The testes are deeply lobed and divided into several distinct pieces lying wholly outside the intestinal caeca. Vitelline glands internal to the intestinal caeca. A large receptaculum seminis present. Parasites in the sting ray.

Type-species :—*Petalodistomum polycladum* Johnston, 1912.

Johnston also included in this genus another species, *Petalodistomum cymatodes*. Travassos (1920) erected the new genus *Staphylorchis*, to accommodate *Petalodistomum cymatodes*, as he considers it more convenient to separate it from the genus *Petalodistomum* and make a new genus of it because it more resembles the genus *Anaporrhutum* than the genus *Petalodistomum*, and he further considers that the disposition of the caeca and of the testes appears to be sufficient for making a separate genus and that less differences exist between *Phylodistomum* and *Catroptroides* on one hand and between *Gorgodera* and *Gorgoderina* on the other.

Diagnosis of the genus *Staphylorchis* Travassos, 1920.

Large Anoporrhutinae with the posterior part more or less rounded. A strong muscular pharynx present, intestinal caeca sinuous but without diverticula. The testes are divided into a large number of small spherical bodies and are external to the intestinal caeca. Vitelline glands between the intestinal caeca. A large receptaculum seminis present. In the body cavity of rays, Australia.

Type-species :—*Staphylorchis cymatodes* (Johnston, 1913), Travassos, 1920.

Diagnosis of the genus *Nagmia* n.gen.

Large Anaporrhutinae with the lateral and posterior borders forming nearly a semi-circle. A muscular pharynx as well as a short oesophagus is present. Intestinal caeca branched. Testes divided into a large number of irregularly-shaped bodies which are definitely outside the intestinal caeca. Vitelline glands between the intestinal caeca. A large receptaculum seminis present.

Type-species :—*Nagmia yorkei*.

This genus most closely resembles the genus *Petalodistomum* Johnston, 1912, but it differs from this mainly in the greater size of the new genus (the size given to *Petalodistomum polycladum* is 3·3 mm. to 3·76 mm. in length, and 3 mm. to 3·5 mm. in breadth ; while that of the new species, *Nagmia yorkei*, is 1·6 cm. to 1·7 cm. in length, and 1·2 cm. to 1·6 cm. in breadth), in the shape of the vitelline glands, which are composed of two sets of small rounded follicles, these sets being close together in the genus *Petalodistomum*, while in the new genus they are composed of two sets each composed of tubular ramifications and are wide apart. Another very important difference is the greater number of the lobules of the testes in the new genus.

The type-species, *Nagmia yorkei*, and specimens of *Anaporrhutum largum* are kept in the Museum of the Liverpool School of Tropical Medicine.

EXPLANATION OF LETTERING

ex.v.	= excretory vesicle.	*r.s.*	= receptaculum seminis.
i.c.	= intestinal caeca.	*t.*	= testes.
ov.	= ovary.	*vt.g.*	= vitelline glands.

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NOTES ON MODES OF RAT INFESTATION WITH *HEPATICOLA HEPATICA*

BY

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Although *Hepaticola hepatica* is the most common nematode parasitic in the liver of the house rat, there is no definite record in the literature of the subject as to the modes of rat infection.

Rate of frequency of the parasite and influence of the season.

Whilst collecting a nematode of the genus *Capillaria* parasitic in the gastric wall of the house rat, the writer studied the frequency of *Hepaticola hepatica* in the same host over a period of three years, from May, 1924, to July, 1927. Almost all the hosts seen were obtained from the bacteriological laboratory of the Osaka municipality, where rats were collected from all parts of the city for examination in connexion with rat plague. Out of 2,222 house rats examined, 1,272 were found to be infected with *H. hepatica* (57·2 per cent.) ; out of 1,189 examined, 809 (68 per cent.) harboured *Cysticercus fasciolaris*.

The graph (p. 110) shows that there is a certain relation between the rate of frequency of the parasite and the seasons, i.e., the lowest rate is in summer, and it increases through autumn and winter, to reach the highest point in spring.

Are the eggs of H. hepatica *passed out of the host with the faeces ?*

It has not yet been ascertained whether the eggs of *H. hepatica* are passed out of the host with the faeces. According to Bancroft, the eggs do not pass out of the host through the intestine ; Railliet, on the other hand, states that he found eggs in the faeces. In Japan, Kikuchi (1923) did not find them, while Kaji (1924) affirms the reverse. Nishigori (1925) is of opinion that they very rarely appear

in the faeces. Iwahashi (1924) found only a single egg, although the material examined was very plentiful. Suda (1928) observed a small number of eggs in the gall bladder of a monkey experimentally infected with *H. hepatica,* and from this fact he deduced the possibility that the eggs pass through the bile-ducts into the intestine.

The writer examined closely almost the entire contents of the digestive canal (stomach and intestines) of twenty-nine house rats collected between September, 1925, and May, 1926, which were simultaneously infected with *H. hepatica* in the liver and *Capillaria bacillata* in the gastric wall, in order to verify the results obtained by other authors. He was unable to find a single egg of *H. hepatica,* while those of *C. bacillata* were common. When the eggs of *H. hepatica* are found it is necessary to discover their origin before deciding that it is a case of infection.

THE RELATION BETWEEN
FREQUENCY OF *H. HEPATICA*
AND THE SEASONS
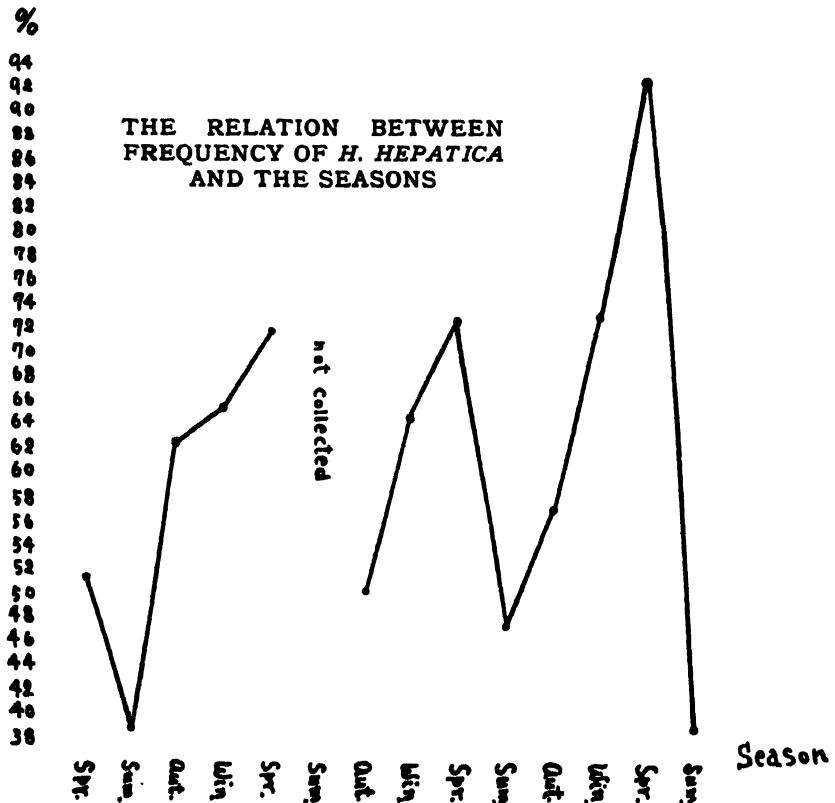
Do the eggs of H. hepatica *appear in the faeces of cats ?*

From September 13th, 1926, to March 3rd, 1927, the writer examined the intestines of 503 cats which were used in exterminating rats in a hide factory in this city, where a large number of cats, dogs and other animals were collected for the manufacture of hides.

The contents of the rectum were examined by the antiformin-ether method for collecting eggs, but only five cases were proved to harbour eggs of *H. hepatica.* The eggs had begun their cell division in one case only, probably because in this instance they had been preserved in antiformin for a few days.

Relation of flies to the dissemination of eggs.

That the fly can be the carrier of parasitic eggs as well as of various kinds of infective germs is already known. From the fact that in summer, flies are often seen in large numbers round the decomposed body of a rat, the writer concluded that the fly might act as an agent in the dissemination of the eggs of *H. hepatica.* He therefore made the following experiments :

Eggs were collected from the livers of rats. An inverted metal net funnel was used, with a diameter of 14 cms. at the base and an aperture of 4 cms. in diameter at the top. A watch glass containing the eggs, together with a little syrupus simplex to attract the fly, was placed inside the net, with two wide glass slides beside it. The faeces deposited on a slide by the flies could thus be easily examined. A certain number of flies were liberated in the net for variable lengths of time. As soon as the slides had been withdrawn, the flies were killed with chloroform. Each fly was washed separately in a centrifuge tube and the water centrifuged to recover any eggs which might have adhered to the fly. The entire intestinal tract of each fly was next examined. The results were as follows :

Out of 83 flies examined in the above experiment, 53, that is 63·9 per cent., carried eggs in the intestinal tract ; the number of eggs found in individual flies varied from 1 to over 1,300 ; the body surface of 79 flies was examined and in 26 cases, that is 32·9 per cent., eggs were present ; the number of eggs found on the surface of the flies varied from 1 to 8, with the exception of one which showed 61 eggs after accidentally being dropped into the watch glass

in which the eggs were collected ; 65 out of 117 faecal spots, that is 55·6 per cent., contained eggs ; from 1 to 65 eggs were found in individual faecal spots. These experiments show that the eggs of *H. hepatica* can be disseminated by the fly.

The development of eggs which had passed through the intestine of the cat.

In the study of the modes of rat infestation with *H. hepatica*, it is important to verify whether the eggs, after passing through the intestine of the cat, are still viable. To establish this point, the writer made the following experiments. Eggs collected from the faeces of a cat fed on the fresh livers of infected rats, were placed in a porous culture dish and kept in the laboratory at a temperature of 26° C. to 35° C. The experiments proved that the power of development of the eggs was not affected by passage through the intestines of the cat.

On the development of eggs passed through the intestinal tract of the fly.

Experiments were made similar to those described in the paragraph entitled ' Relation of flies to the dissemination of eggs.' To cultivate the eggs in the faecal spots, the glass slides were kept moderately moist in the incubator.

Control.

Eggs collected from the liver of infected rats on October 22nd, 1927, were cultured on October 27th, 1927. Twenty-three days after cultivation, mature eggs were observed. From the results of these experiments it would appear that, as in the case of the cat, the power of development of the eggs is not affected by passage through the intestinal tract of the fly.

The influence of desiccation on the eggs.

Eggs were smeared on a number of small pieces of gauze, and after being kept in the laboratory at a temperature of from 6° C. to 36° C., were placed in a porous culture dish in the incubator at a temperature of from 27° C. to 30° C. Eggs dried for twenty-nine days matured ; eggs dried for thirty-one or forty-one days were found to be affected, while those dried for forty-eight days were dead.

CONCLUSIONS

1. *H. hepatica* is the most common nematode parasitic in the liver of the house rat, and the infestation percentage is 57·2.

2. The rate of frequency of the parasite and the season appear to be related, the rate being at its lowest in summer and increasing through autumn and winter, until it reaches its highest in the succeeding spring.

3. No eggs were found in the intestines of twenty-nine infected rats examined by the writer.

4. It appears that flies may be capable of disseminating eggs.

5. The power of development of eggs is not affected by passing through the intestinal tract of the cat or of the fly.

6. The eggs of *H. hepatica* dried for about one month, do not lose their power of development.

7. The writer believes that the fly is the chief agent in the dissemination of these eggs.

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STUDIES IN CHEMOTHERAPY[*]
II. THE ACTION *IN VITRO* OF NORMAL HUMAN SERUM ON THE PATHOGENIC TRYPANOSOMES, AND ITS SIGNIFICANCE

BY

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AND

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HISTORICAL

In the year 1902, Laveran made the interesting discovery that normal human serum, when injected into mice suffering from nagana, exerted a marked effect on the course of the infection; and the phenomenon has since been the subject of a vast amount of work. Laveran and Mesnil (1902) found that when serum is heated to 56° C. for an hour it loses about half of its activity, and that heating to 62° C. suffices to render it almost inactive. This was confirmed by Goebel (1907), and later by Rosenthal and Freund (1923), Adams (1928), and others; and it is now generally agreed that heating to 64° C. completely destroys the trypanocidal power of human serum, and that it cannot be restored by the addition of complement. Laveran and Mesnil found that human serum retains its trypanocidal power for some time when it has been collected aseptically, but serum in which moulds or bacteria have developed rapidly loses its activity: dried human serum retains the properties of the liquid serum for at least six months. These observations were confirmed by Goebel, by Leboeuf (1911), and quite recently by Adams (1928) and by Pfannenstiel and Scharlau (1929).

Laveran and Mesnil concluded that the action of human serum could not be studied *in vitro*, because trypanosomes suspended in

fresh human serum lived as long as in human serum heated to 62° C., or in the inactive sera of other animals : a similar conclusion was reached by Goebel (1907). Notwithstanding, however, their inability to demonstrate a trypanocidal action of human serum *in vitro*, Laveran and Mesnil considered that there is a close relationship between the immunity of man to nagana and this peculiar property of his serum. They remark that a single experiment indicated that human citrated plasma is but slightly active as compared with serum, but it was inferred that the plasma must contain sufficient active principles to protect the organism against invasion by trypanosomes, although it does not possess sufficient activity to be curative when injected into infected mice. It was further considered that the active substance was produced by the leucocytes, and that this was the explanation of the fact that fluids from serous cavities— pleural effusion and ascitic fluid—which contain few leucocytes may be almost inactive.

Salmon (1910), who endeavoured to trace the origin of the trypanocidal substance, injected the leucocytes of two kinds of pus, and an extract of lymphatic glands, into mice infected with nagana, without result. He states that the trypanocidal substance is present in the fluid portion of the blood only, and that the active substance does not penetrate through the meninges as the cerebro-spinal fluid is inactive ; nor does it traverse the kidneys, for in two instances albuminous urine was ineffective. In contrast to Lavèran and Mesnil, it was found by Salmon that serous exudations, such as pleural fluid and hydrocoele fluid, were very active. Quite recently Pfannenstiel and Scharlau (1929) have recorded that they also were unable to obtain any active substance from the formed elements of the blood.

Jacoby (1909), having found that certain very lipaemic sera were powerfully trypanocidal, showed that the portion of the serum insoluble in ether contained all the active principle. Goebel (1907) found that the active-substance belonged to the globulin. fraction and was precipitated by magnesium sulphate ; and his work was confirmed and extended by Rosenthal and Freund (1923a), who proved that the trypanocidal substance is bound up with the euglobulin and pseudoglobulin fractions of human serum, and is not contained in the fibrinoglobulin or albumin fractions.

Laveran (1904a) discovered that the serum of baboons (*Papio anubis* and *Papio cynocephalus*) possessed a trypanocidal power similar to that of human serum. The trypanocidal action of the serum of primates was carefully re-investigated by Mesnil and Leboeuf (1910), who found that the species with active serum could be arranged thus in descending order :—baboon, man, mangabey, and mandrill : the sera of *Macacus, Cynomolgus, Cercopithecus* and of the anthropoid apes were inactive. When the serum of the baboon was tested on the various pathogenic trypanosomes, it was found that these could be arranged in two well-defined classes :— *T. brucei, T. evansi, T. togolense, T. equinum,* and *T. pecaudi,* which were very sensitive ; and *T. gambiense, T. dimorphon,* and *T. congolense,* which were hardly sensitive at all. The following year an unexpected discovery was made by Mesnil and Ringenbach (1911), who found that human serum exercised a marked effect on *T. rhodesiense* infections in mice.

As was pointed out by Laveran and Mesnil (1904), the injection of human serum into infected mice causes, as a general rule, only a temporary disappearance of the parasites from the blood ; after a variable time they reappear, and require a fresh injection of serum to cause them to disappear again. It was found that human serum, frequently injected into an infected animal, lost its effectiveness upon the trypanosomes very slowly, and that an animal could be successfully treated in this way for two or three months. Jacoby (1909) succeeded in producing a strain of nagana resistant to 2 c.c. of human serum, which he considered to be the maximum dose which a mouse will tolerate. Leboeuf (1911) attempted to confirm the work of Jacoby, and although he several times obtained mice in which the trypanosomes were resistant to one or even two successive 2 c.c. doses, he never succeeded in transmitting the refractory state to further mice. Laveran and Nattan-Larrier (1912) showed that *T. rhodesiense* acquired somewhat rapidly a certain degree of resistance to human serum ; and, in a later paper (1912a), that two serum-resistant strains lost their resistance after, in one case 57 to 73 mouse passages, and in the other case 25 to 40 mouse passages. In similar experiments performed by Mesnil (1912a) the resistance was lost about the 30th passage.

Jacoby (1909) put forward the suggestion that strains of nagana

resistant to human serum might be pathogenic to man ; Mesnil and Leboeuf (1912), however, failed to infect a *Cynocephalus* with a strain of nagana, which in the mouse resisted 2 c.c. of the serum of this animal. Recently Collier (1924) made a strain of *T. brucei* so resistant to human serum that infected mice resisted a dose of 2·5 c.c., that is twenty-five times the dose which sufficed to clear the blood of mice infected with the normal strain ; he inoculated himself and subsequently four other persons with the resistant strain with negative results.

Laveran and Mesnil (1912) observed great variation in the trypanocidal power of serum from different individuals. They found that the serum of adults was much more active than that of the new-born child ; and they remark that while Ehrlich (1907) had observed that the serum of individuals with disease of the liver was but slightly active, Neumann (1911) had found that the reverse was the case with the serum of women after child-birth.

Of recent years this aspect of the subject has attracted much attention. The trypanocidal properties of the sera of a mother and her new-born child were examined by Rosenthal and Kleeman (1915) ; the former exhibited trypanocidal action in normal degree, whilst in the latter the trypanocidal action was almost completely absent. Platau (1916) showed that the titre of trypanocidal substance varied considerably in the sera of apparently healthy individuals, and that frequently it was greatly diminished in persons suffering from diseases of the liver. Rosenthal and Krueger (1921), and Rosenthal and Nossen (1921), showed that the trypanocidal power of human serum was greatly decreased, not only in cases exhibiting marked lesions of the liver without jaundice, but also in cases of mechanical jaundice : further work indicated that the disappearance of the trypanocidal power of the serum in the latter condition was not due to bile constituents. The conclusion was reached that the trypanocidal power of human serum stands in the closest relationship with the functional activity of the liver,· and that in all probability the liver is the place of its formation. Zeiss (1921) likewise found that icteric human serum was without trypanocidal action, but that the sera of two cases of Hodgkin's disease behaved exactly as normal serum. Leichtentritt and Zielaskowski (1922) showed that in children in the acute stage of Barlow's disease, and in

those suffering from deficiency diseases, the trypanocidal activity of the serum was diminished. Mignoli (1924) examined the trypanocidal action of the sera of twenty-five patients suffering from such diseases as tuberculosis in various stages, carcinoma of the head of the pancreas with marked jaundice, pancreatic diabetes, catarrhal jaundice, bronchopneumonia, enterica, encephalitis lethargica, and cardiac valvular lesions : he found that the serum of a case of advanced tuberculosis was less trypanocidal than that from an early or improving case, that the presence of malignant disease did not affect the trypanocidal action, but that liver conditions altered it in the degree to which the liver cells were injured by disease. Grünmandel and Leichtentritt (1924) found that in conditions which are dependent on lack of vitamins the trypanocidal action of serum is decreased or even absent : investigations made on children suffering from corneal ulcers, a condition which, in 1923, reached unprecedented proportions owing to the lack of high-grade fats and the poor quality of the milk, showed that the serum exhibited a complete absence of trypanocidal substance ; similar observations were made on children suffering from alimentary oedema resulting from improper dietary. Peutz (1922) found that the trypanocidal power of human serum may diminish or disappear in pathological conditions, but no fixed rules could be formulated which might render this phenomenon of diagnostic importance. These observations were extended by Leichtentritt the following year. Munter (1925) found that in cases of diffuse parenchymatous degeneration of the liver the diminution of trypanocidal power of the serum was exactly parallel to the severity of the disease. Opitz and Zweig (1925), Leichtentritt and Opitz (1927), and Leder (1928), observed that the serum of cases of haemophilia was deficient in trypanocidal power ; but this was not confirmed by Werner and Hartmann (1926).

Neumark and Pogorschelsky (1925) found that the serum of healthy sucklings possessed no detectable amount of trypanocidal substance during the first few weeks of life, but that as age increases these substances appear and are well-developed at the end of three months : they further found that any infection is sufficient to cause a decrease of trypanocidal substance. Levy (1928), who repeated the work of Neumark and Pogorschelsky, found that

trypanocidal substance was present in all the infants examined by him at the third week of life, and that atrophic conditions and infections could not be demonstrated unequivocally to exert any influence on the trypanocidal substance. Nattan-Larrier and Lépine (1927) examined the sera of twelve women and of twelve new-born children and found that those of the mothers were always active, whereas those of the children were rarely active and even then only feebly so.

The ever-growing interest in the trypanocidal action of human serum has caused Rosenthal and his colleagues to devote much time, during the past eight or nine years, to an investigation into the mechanism of its action. Rosenthal and Freund (1923a) injected rabbits four times intravenously with human serum and produced thereby a potent precipitating immune serum—1 c.c. of 1 : 10,000 dilution of the human serum giving a definite precipitate with 0·1 c.c. of the immune serum. This immune serum, when incubated with human serum in the proportion of 2 c.c. of immune serum to 0·2 c.c. of human serum, failed to prevent the prophylactic action of the latter when the mixture was tested on nagana mice. From this Rosenthal and Freund conclude that the trypanocidal substance of human serum possesses no antigenic qualities; and furthermore that, in contrast to the finding of Goebel (1907), contact with a heterologous serum does not weaken its trypanocidal power. It was next found that in mice which had been given four preliminary injections of 0·5 c.c. of human serum, subcutaneously or intra-peritoneally at four-day intervals, human serum exerted no trypano-cidal action when it was tested eight days after the last of the four preliminary injections. Further work showed that this preventive action of preliminary injections of human serum had nothing to do with any red cell or leucocyte protein it might have contained; and also that the preventive effect was only obtained by preliminary injections of human serum and not of rabbit serum. Subsequent work showed that the blood of mice which had been given four similar injections of human serum did not, when mixed *in vitro* with human serum and kept for two hours at 37° C., destroy the trypanocidal power of the latter. From this and other work Rosenthal and Freund concluded that the inhibition of the thera-peutic action of human serum in mice which had received preliminary

injections of human serum was not an immunity phenomenon depending on the production of anti-trypanocidal substance by the mouse, but an exhaustion phenomenon involving the elimination of something in the treated animal, the function of which is to form trypanocidal substances out of human serum. Reference is then made to the fact that Laveran and Mesnil had found that human serum had no trypanocidal action *in vitro*, and that the so-called trypanocidal substances of the serum were unable to become anchored *in vitro* to the trypanosomes. Rosenthal and Freund record that, as the result of their own observations, they were able fully to confirm these statements. Trypanosomes suspended in human serum showed neither as regards motility nor morphology any differences from those suspended in physiological saline ; and parasites, which had been many hours in contact with human serum, and then centrifuged and washed free from serum, readily produced infection when inoculated into a mouse. These considerations led Rosenthal and Freund to the conclusion that trypanocidal substance does not exist as such in human serum, but as a trypanocidogenous substance which on injection is converted into trypanocidal substance by a ferment supplied by the injected animal. This they consider to be the explanation of the considerable period which elapses before trypanocidal action is seen in the blood of the treated mouse. During the conversion of trypanocidogenous into trypanocidal substance the ferment is consumed, and hence is explained the negative result which attends the serum treatment of animals which have previously received large doses of human serum.

These researches were continued by Rosenthal and Spitzer (1924), who investigated the mechanism whereby human serum, which is inactive *in vitro*, is converted in the animal body into an active trypanocidal substance. The starting-point of these investigations was the observation that, after simultaneous intraperitoneal injection into mice of human serum and trypanosomes, evidence could be obtained, by sampling the peritoneal contents, of a marked trypanocidal action going on locally in the peritoneal cavity. The fact that the intraperitoneal injection of the mixture of trypanosomes and human serum resulted in a great exudation of leucocytes—first polymorphonuclears and later macrophages—caused the authors to enquire whether these cells might not secrete the ferment necessary

for the conversion of the trypanocidogenous substance of human serum into the trypanocidal substance. This view had, however, to be abandoned, as mice rendered aleucocytic by intense treatment with thorium X behaved as normal mice, when subsequently infected with nagana and treated with human serum. Rosenthal and Spitzer then passed to an enquiry whether the reticulo-endothelial system of the mouse played any part in the curative mechanism. They found that iron-blocking of the reticulo-endothelium alone often sufficed to produce a distinct weakening of the trypanocidal effect of human serum, that a greater decrease followed splenectomy, and finally that combined iron-blocking and splenectomy resulted in a pronounced diminution in the action of human serum. From these observations the authors conclude that the reticulo-endothelial system of the mouse is responsible for the conversion of the inactive trypanocidogenous substance in human serum into the active trypanocidal substance ; and they further explain the observations of Rosenthal and Freund (1923a),—that human serum has no therapeutic action in mice which have received before infection preliminary injections of human serum—on the hypothesis that the preliminary injections of serum exhaust the reticulo-endothelial system of the mouse, so that it gradually becomes unable to convert trypanocidogenous into trypanocidal substance.

In his latest communication Rosenthal (1929) deals with the distribution, transformation, and destruction of the trypanocidogenous substances of human serum in the animal body. He traced the distribution of the trypanocidogenous substance in the bodies of guinea-pigs twenty minutes after intravenous injection of 4 c.c. of human serum, and found that the great proportion of what was injected was still in the blood of the guinea-pig, extracts of the liver, spleen, muscle and kidneys containing practically none. Similarly, in mice which had received 2 c.c. of human serum intra-peritoneally, the trypanocidogenous substance was found twenty-four hours later to be mainly in the blood of the mouse, relatively little was found in the peritoneal fluid and none in the organ extracts. Further analysis showed that the trypanocidogenous substance was limited to the plasma of the mouse and was not bound to the erythrocytes. Rosenthal then concerned himself with an attempt

to demonstrate the presence of the actual trypanocidal substance in the blood and peritoneal fluid of mice injected intraperitoneally twenty-four hours previously with 2 c.c. of human serum. The citrated blood and peritoneal fluid of the treated mice were incubated at 35° C. in the absence of air for three and a half hours with a few drops of the citrated blood of a heavily infected mouse : the mixtures were then centrifuged, the supernatant fluids removed and the deposits washed thrice with saline. The washed deposits, and the supernatant fluids to which were added a few drops of a trypanosome suspension, were then injected into a series of mice. The results of these experiments showed that, as a rule, the washed deposits infected, and the supernatant fluids + trypanosomes did not infect. In only two experiments did the deposits fail to infect. From these observations Rosenthal concluded that only exceptionally could the existence of the intravitally formed trypanocidal substance be demonstrated *in vitro*.

In another series of experiments, Rosenthal endeavoured to trace the distribution of the trypanocidogenous substance in the bodies of two mice, each of which had been injected with 2 c.c. of human serum (40 therapeutic units in all). He found that after six hours 8 units were in the blood of the mice and 24 in the peritoneal fluid, after twenty-four hours 16 units in the blood and 8 in the peritoneal fluid, and after forty-two hours 3 in the blood and only a trace in the peritoneal fluid ; in no instance was any trypanocidogenous substance found in the urine or organ extracts.

From all this work Rosenthal concludes that the inactive trypanocidogenous substance present in human serum is characterized from its biological behaviour in the animal body by :—its relatively slow conversion, its prolonged sojourn and pronounced concentration in the circulation, its slight tendency to escape into the tissues, and its failure to appear in the urine. It is broken down in the metabolic processes of the animal body and from it is formed the trypanocidal substance, the most characteristic feature of which is the difficulty with which its presence can be demonstrated under the conditions of *in vitro* experiments.

EXPERIMENTAL

In view of the almost* unanimous agreement—Laveran and Mesnil (1902), Goebel (1907), Braun and Teichman (1912), Rosenthal and Freund (1923a), Adams (1928), and Pfannenstiel and Scharlau (1929)—that human serum exercises *in vitro* no harmful action on the pathogenic trypanosomes, it was with considerable surprise that we found, as already mentioned in our previous paper (1929), that normal human serum rapidly destroyed *T. rhodesiense* and *T. equiperdum in vitro* at 37° C. The subject seemed to us to be so important, and possibly to have such a far-reaching bearing on the epidemiology of human trypanosomiasis, that we decided to reinvestigate the whole question of the action *in vitro* of human serum on pathogenic trypanosomes.

In numerous experiments the action of various normal human sera on *T. equiperdum*, two strains of *T. congolense*, *T. rhodesiense*, and *T. gambiense*, and a strain obtained from a case of sleeping sickness in N. Nigeria, viz. :—' Sherifuri K ' strain,† was examined by means of the technique previously described. The blood of an infected mouse was so diluted in Ringer-glucose‡ solution that when 1 vol. was added to 19 vols. of human serum the concentration of trypanosomes was about 1,000 per cmm. The mixtures were placed in the usual tubes and kept in the water-bath at 37° C. The results of these experiments are summarized· in Table I, from which it will be seen that *T. equiperdum*, *T. rhodesiense*,

* Saito (1927) briefly states in the German summary of his Japanese paper that human serum had a definite trypanocidal action *in vitro* on a strain of *T. gambiense* which had been preserved in mice for years.

† The history of these strains is as follows :—
T. equiperdum : origin unknown, but has been maintained for many years in European laboratories by passage through mice.
T. congolense H. : this parasite was received from Wenyon to whom it was sent from East Africa by Mr. Hornby in June, 1929, and has since been maintained by passage through various laboratory animals—1 rabbit, 3 rats and 2 guinea-pigs.
T. congolense S. : this parasite was obtained from a sheep naturally infected in Sierra Leone and brought to Liverpool in November, 1929.
T. rhodesiense : isolated from man in 1923, and since maintained by passage through mice.
T. gambiense : isolated from a Nigerian case of sleeping sickness in March, 1922, and since maintained by passage through mice.
' Sherifuri K ' strain : isolated from the gland juice of a case of sleeping sickness at Sherifuri, N. Nigeria, on 22 February, 1929, and since maintained by passage through a series of five guinea-pigs. The strain is peculiar in that it exhibits numerous posterior-nuclear forms.

‡ Sodium chloride ... 0·9 gm.
Potassium chloride ... 0·025 gm.
Calcium chloride ... 0·02 gm.
Sodium bicarbonate ... 0·015 gm.
Glucose ... ... 0·2 gm.
Distilled water... ... 100·0 c.c.

TABLE I

Showing the trypanocidal action of normal human serum *in vitro* at 37° C. on various pathogenic trypanosomes

Individual	Period of time (in hours) in which at least 95% of the parasites were destroyed				
	Date	*T. rhodesiense*	*T. equiperdum*	*T. congolense*	*T. gambiense*
A.R.D.A. ...	10.10.29	2	2	...	No action in 12 hours
	15.10.29	2	...	...	...
	5.2.30	2	...	2	No action in 24 hours
W.J.C. ...	22.10.29	...	2	...	...
A.C. ...	9.9.29	2	...	...	...
	10.9.29	2	...	...	...
	15.9.29	2	...	...	...
T.D. ...	23.12.29	2	2	...	...
D.D. ...	13.9.29	1½	2	...	...
	17.9.29	2	...	...	No action in 18 hours
	19.9.29	2	...	...	...
	30.9.29	2	...	...	...
	15.10.29	2	...	...	...
	5.2.30	...	...	2	...
A.J. ...	27.8.29	1½	...	...	...
	28.8.29	2	...	...	No action in 4 hours
W.Y. ...	28.8.29	2	...	...	No action in 18 hours
A.M. ...	24.9.29	2	...	...	No action in 5 hours
	30.9.29	2	...	...	...
	15.10.29	2	...	...	...

TABLE I—*Continued.*

Individual	Period of time (in hours) in which at least 95% of the parasites were destroyed.				
	Date	*T. rhodesiense*	*T. equiperdum*	*T. congolense*	*T. gambiense*
F.M. ...	10.10.29	2	2	...	No action in 12 hours
	15.10.29	2	...	...	...
	13.1.30	1	...	...	...
	5.2.30	1	...	2	No action in 24 hours
H.F.N. ...	10.10.29	2	2	...	No action in 12 hours
	15.10.29	2	...	...	...
G.P.I. ...	21.8.29	...	2	...	...
	17.9.29	2	...	...	No action in 18 hours
	19.9.29	2	...	...	...
A.P. ...	4.9.29	...	1	...	...
A.S. ...	17.9.29	2	...	...	No action in 18 hours
	19.9.29	2	...	...	...
	20.9.29	1½	...	...	...
	21.9.29	1	...	...	...
B.S. ...	10.10.29	2	2	...	No action in 12 hours
C.S. ...	27.8.29	1	...	...	No action in 4 hours
	28.8.29	2	...	...	...
	19.9.29	2	...	...	...
	20.9.29	1	...	...	...
	21.9.29	1	...	...	...
	24.9.29	1½	2	...	No action in 15 hours
	30.9.29	1½	...	...	...

N.B. As a rule the first reading was not made until after the lapse of two hours.

and *T. congolense* are quickly destroyed by normal human serum *in vitro* at 37° C., and that *T. gambiense* is practically unaffected by it. Reference to Table XII shows that the ' Sherifuri K ' strain was, in contradistinction to the old laboratory strain of *T. gambiense*, definitely affected by normal human serum, but to a much less extent than was the old laboratory strain of *T. rhodesiense*. The same results were obtained in those cases in which citrated human - plasma was employed instead of serum. The cytolytic action of human serum on the trypanosomes is very characteristic and can easily be observed under the microscope. The first obvious change is that the parasites become less refractile, and this is quickly followed by vacuolation and granulation of the cytoplasm. Soon the trypanosomes lose their characteristic form and become tadpole- or racquet-shaped ; these in turn become ring-shaped and finally completely disintegrate, so that eventually nothing remains to be seen.

The process is so constant and so striking that it is amazing that it has hitherto escaped observation. Doubtless this is mainly due to the inability of previous workers to keep pathogenic trypanosomes alive *in vitro* at 37° C. for any length of time. We have repeated our experiments on the trypanocidal action of human serum, modifying the technique only in so far as the experiments were conducted at laboratory temperatures instead of at 37° C. The results of this work, which are summarized in Table II, show clearly the enormous influence which temperature exercises on the reaction, trypanolysis occurring much more rapidly at 37° C. than at 15° C.

Attention has been frequently drawn to the fact that human serum loses its power to protect and cure infected mice if it is heated to from 62° C. to 64° C. The effect of heat on the power of human serum to destroy trypanosomes *in vitro* was examined, and the results are shown in Table III, from which it is seen that heating to 64° C. for 30 minutes suffices to destroy the trypanocidal action of human serum *in vitro*, and that heating to 60° C. for a similar period partially destroys its action.

TABLE II.

Showing the trypanocidal action of normal human serum *in vitro* at 37° C. and 15° C. respectively.

Experiment	Serum	Temperature of experiment	Number of living trypanosomes (*T. rhodesiense*) per 256 squares of the haemocytometer scale						
			Start	1 hour	2 hours	4 hours	6 hours	9 hours	24 hours
1	Human A	37° C.	84	1*	0	...	...	...	...
		15° C.		64	...	64	5*	0	...
	Sheep	37° C.		...	...	...	...	...	61
2	Human B	37° C.	44	14*	0	...	...	...	...
		15° C.		34	26*	26*	18*	13*	0
	Sheep	37° C.		...	...	...	...	...	40
3	Human C	37° C.	68	0	...	...	...	...	...
		15° C.		65	50*	35*	21*	1*	...
	Sheep	37° C.		...	...	...	...	65	53

* Dead and disintegrating trypanosomes seen.

TABLE III.

Showing the effect of heat on the trypanocidal power of human serum *in vitro*.

Experiment	Case	Temperature to which serum heated	Number of living trypanosomes (*T. rhodesiense*) per 256 squares of the haemocytometer scale							
			Start	1 hour	2 hours	4 hours	6 hours	8 hours	10 hours	24 hours
1	G	Unheated	50	0	...	...	...	...	...	...
		56° C. for 30 mins....		0	...	...	...	...	...	...
		60° C. ,, ...		33	13*	8*	3*	1	...	...
		64° C. ,, ...		59	63	59	62	66	...	34
2	C	Unheated	35	10*	4*	0	...	...	...	...
		64° C. for 20 mins....		34	36	40	...	...	13	...
3	S	Unheated	54	3*	0	...	...	...	...	...
		64° C. for 20 mins....		45	52	30	...	...	...	0
4	M	Unheated	70	0	...	...	... ↖	...	' ...	...
		64° C. for 30 mins....		64	62	...	...	77	...	31

* Dead and disintegrating trypanosomes seen.

It is further seen from Table IV that the addition of fresh rabbit serum fails to restore the lost trypanocidal power of human serum, which has been heated to 64° C.

TABLE IV.

Showing the failure of fresh rabbit serum to reactivate the trypanocidal power of human serum heated to 64° C.

Tube		Number of living trypanosomes (*T. rhodesiense*) per 256 squares of the haemocytometer scale					
		Start	1 hour	3 hours	5 hours	17 hours	30 hours
1	Human serum, unheated, 1 c.c.	⎧	0	...	...	...	...
2	Human serum, 56° C. for 30 mins., 1 c.c. ...		0	...	...	...	...
3	Human serum, 60° C. for 30 mins., 1 c.c. ...		10*	1*	0	...	...
4	Human serum, 64° C. for 30 mins., 1 c.c. ...	24 ⎨	30	30	30	19	13
5	Human serum, 64° C. for 30 mins., ·5 c.c. + fresh rabbit serum, ·5 c.c.		33	30	30	24	12
6	Human serum, unheated, ·5 c.c. + fresh rabbit serum, ·5 c.c.		1*	0	...	...	...
7	Fresh rabbit serum, 1 c.c.	⎩	...	30	27	21	14

* Dead and disintegrating trypanosomes seen.

These preliminary experiments thus showed that normal human serum contains a trypanocidal substance which is active *in vitro* at 37° C., but is much less active at laboratory temperatures ; and furthermore, that the trypanocidal substance is destroyed by heating to 64° C. for 30 minutes, and that it cannot be restored by the addition of fresh rabbit serum.

Attention was now directed to an investigation of the amount of trypanocidal substance in normal human serum, in order that it might be possible to contrast the trypanocidal power of various normal sera, and of the sera of individuals suffering from various diseases ; and also that we might be enabled to obtain some light on the mechanism of the curative action of human serum in mice infected with trypanosomes.

The determination of the trypanocidal titre of human serum, although at first sight a perfectly simple procedure, proved in point of fact to be a matter of very considerable difficulty. It is

obvious that theoretically this enquiry could be made in two directions : firstly, by experiments in which the concentration of the trypanosomes was constant and that of the human serum varied ; and secondly, by experiments in which the concentration of human serum was constant and that of the trypanosomes varied. In following the first line of enquiry the difficulty which confronted us was the discovery of a medium which, while it sufficed to support the trypanosomes alive at 37° C. for the length of time necessary to judge of the action of human serum, viz., twenty-four hours, would not react with human serum so as to inhibit or destroy its trypanocidal power. As we have shown in our previous paper, a certain concentration of serum (sheep, rabbit, etc.) is essential for the support of the pathogenic trypanosomes, and if the concentration falls below this point the parasites rapidly succumb from arthrepsis. Preliminary investigations with rabbit or sheep serum diluted with Ringer-glucose solution soon showed that when the concentration of serum fell below about 1 in 25, the parasites succumbed in a few hours at 37° C. This degree of dilution consequently set a limit to what could be done in determining the trypanocidal titre of human serum by the simple and direct method of adding a definite number of trypanosomes to equal volumes of Ringer-glucose solution containing varying concentrations of human serum.

A number of experiments of this nature were performed ; in these, human serum, human plasma, human serum heated to 64° C. for thirty minutes, sheep serum and sheep plasma, were all diluted to varying extents up to 1 : 25 with Ringer-glucose solution. To equal volumes of each of these varying dilutions of serum were added 1/20th vols. of a trypanosome suspension, and the trypanocidal effect observed at 37° C. and 15° C., respectively.

The results of a typical experiment are shown in Table V, from which there clearly emerges a number of facts.

1. Trypanosomes suspended in Ringer-glucose solution die rapidly.*

2. The same trypanosome suspension survives well for at least nine hours in Ringer-glucose solution provided it contain 1/25th of its volume of sheep serum ; and if the concentration

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* This is especially the case if, as the result of sterilization, hydrolysis of the NaHCO₃ occurs.

TABLE V.

Showing the trypanocidal action *in vitro* of human serum and of human plasma diluted to various extents with Ringer-glucose solution at 37° C. and 15° C. respectively.

Temp. of experiment		Concentration of serum or plasma in Ringer-glucose solution	Number of living trypanosomes (*T. rhodesiense*) per 256 squares of the haemocytometer scale						
			Start	1 hour	2 hours	3 hours	6 hours	9 hours	24 hours
37° C.	Human serum, unheated	Pure		0	...	...	...	...	...
		1 : 2		1*	0	...	...	...	...
		1 : 5		3*	0	...	...	...	...
		1 : 10		4*	0	...	...	...	...
		1 : 15		9*	0	...	...	...	...
		1 : 20		4*	0	...	...	...	...
		1 : 25		13*	0	...	...	...	..
	Human serum, heated to 64° C. for 30 mins.	Pure	64	...	...	...	...	86	31
		1 : 2		...	62	...	44	77	42
		1 : 5		...	...	...	61	...	33
		1 : 10		...	89	...	...	...	26
		1 : 15		...	70	...	...	63	23
		1 : 20		...	61	...	...	55	8
		1 : 25	55	...	62	...	58	23*	0
	Sheep serum	Pure		...	...	...	...	65	53
		1 : 2		...	...	...	...	...	84
		1 : 5		...	...	...	...	64	86
		1 : 10		...	...	...	...	...	0
		1 : 15		...	...	...	...	76	4
		1 : 20		...	...	85	...	...	0
		1 : 25	77	...	...	63	64	82	0
	Ringer-glucose	...	31	0	...	...	...	...	...
	Human plasma, unheated	1 : 2	70	0	0	...	...	...	...
		1 : 5		1*	0	...	...	...	...
		1 : 10		2*	0	...	...	...	...
		1 : 15		4*	0	...	...	...	...
		1 : 20		21*	0	...	...	...	...
		1 : 25		8*	0	...	...	...	...
	Sheep plasma	1 : 2		...	...	...	...	86	80
		1 : 5		...	...	...	...	...	82
		1 : 10		...	...	62	...	78	28
		1 : 15		...	...	60	...	53	0
		1 : 20	84	...	...	56	...	...	0
		1 : 25		...	84	57	...	18	0
	Citrated Ringer-glucose	...	69	0	...	...	...	...	...
15° C.	Human serum, unheated	Pure	65	...	46	21	1	...	...
		1 : 2		...	43	6	7	...	...
		1 : 5		...	...	32	11	...	0
		1 : 10		...	63	52	15	...	...
		1 : 15		...	...	59	39	...	5
		1 : 20		...	63	62	43	...	9
		1 : 25		...	77	67	39	...	6
	Human serum, heated to 64° C. for 30 mins.	Pure		...	...	...	53	86	100
		1 : 25		...	...	...	107	94	88

* Dead and disintegrating trypanosomes seen.

of sheep serum be increased to 1 : 5 the parasites survive for at least twenty-four hours.

3. The trypanosomes suspended in the various dilutions of human serum in Ringer-glucose solution were all dead within about two hours, even when the concentration of human serum was only 1 : 25.

4. That this was not due to arthrepsis but to a definite trypanocidal action of the human serum is shown by the fact that in the various dilutions of human serum, which had been heated to 64° C. for thirty minutes, the trypanosomes survived practically as well as in the corresponding dilutions of sheep serum.

5. Human plasma and sheep plasma behaved as did their respective sera. No evidence was obtained that, within the limits of the experiment, the trypanocidal power of human plasma was inferior to that of human serum.

6. The importance of temperature in the reaction of human serum and trypanosomes is shown by the fact that at 37° C. all the trypanosomes, even in the greatest dilutions of human serum, were dead within two hours, whilst at 15° C., even in the highest concentrations, a proportion of the parasites was alive after nine hours, and in the lower concentrations even after twenty-four hours.

From experiments of this nature, we reached the general conclusion that, under these conditions of experiment, with a suspension of about 1,000 trypanosomes per cmm., the trypanocidal action of human serum and plasma can be demonstrated up to a dilution of 1 : 25. For reasons to which we have already referred, we were compelled, when we wished to examine the trypanocidal action of higher dilutions of human serum, to use as a diluent some fluid which itself is a nutrient medium capable of supporting the trypanosomes for a sufficient period to enable us to judge of the action of various concentrations of human serum added to it. Unfortunately, as we have already mentioned, all such media must—so far as we are at present aware—contain the serum of rabbit or sheep, or of some other animal, as an essential constituent. In our earlier attempts to determine the trypanocidal titre of human serum by diluting with such nutrient media we were much troubled by anomalous and apparently contradictory results.

In some of our experiments, as in that outlined in Table VI,

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TABLE VI

Showing the trypanocidal action of human serum diluted to various extents with nutrient medium consisting of sheep serum (heated to 60° C.) and Ringer-glucose solution.

Sheep serum	Human serum	Concentration of human serum in nutrient medium (equal parts of sheep serum and Ringer-glucose solution)	Number of living parasites (T. rhodesiense) per 256 squares of the haemocytometer scale			
			Start	2 hours	4 hours	8 hours
F	S	Pure human		0	...	...
		1 : 2		0	...	...
		1 : 5		1	...	...
		1 : 10		0	...	...
		1 : 20		1	0	...
		1 : 40		5*	0	...
		1 : 80		3*	0	...
		1 : 160		7	0	...
		1 : 320		9*	5*	...
		1 : 640		28*	5*	...
		1 : 1280		30*	13	9
		1 : 2560		43	59	20
		1 : 5120		48	42	39
		1 : 10240		50	64	70
	M	Pure human	50	1*	0	...
		1 : 2		7	0	...
		1 : 5		1	0	...
		1 : 10		7	2	...
		1 : 20		3	2	...
		1 : 40		3	1	...
		1 : 80		7	0	...
		1 : 160		4	1	0
		1 : 320		10	4	2
		1 : 640		18	8	4
		1 : 1280		33	24	78
		1 : 2560		58	52	27
		1 : 5120		49	54	66
		1 : 10240		53	62	69
	D	Pure human		3	...	...
		1 : 2		6	...	...
		1 : 5		0	...	...
		1 : 10		0	...	..
		1 : 20		2	...	...
		1 : 40		0	...	...
		1 : 80		...	3	0
		1 : 160		...	1	2
		1 : 320		...	3	1
		1 : 640		...	16	4
		1 : 1280		...	28	6
		1 : 2560		...	40	19
		1 : 5120		...	47	40
		1 : 10240		...	52	70
		Sheep serum + Ringer-glucose		50	72	75

' Dead and disintegrating trypanosomes seen.

we obtained apparently quite definite and clear-cut evidence that the serum of normal individuals exhibited pronounced trypanocidal action *in vitro* within a period of four to eight hours, at 37° C., even when diluted 2,500-5,000 times.

In other experiments of a similar nature, however, we obtained entirely different results, little evidence of trypanocidal action being seen with comparatively great concentrations of human serum. Moreover, remarkable and puzzling paradoxes were at times encountered in which the trypanosomes remained alive in the presence of relatively great concentrations of human serum, and were destroyed in much lower concentrations in the same medium (equal parts of deactivated sheep serum and Ringer-glucose solution).

These anomalous results caused us to investigate the question whether the difference lay in the fact that human serum from different normal individuals, or from the same individual at different times, exhibited varying degrees of trypanocidal power ; or whether the discrepancies depended on the fact that in the different experiments the sera of different sheep were used as the diluent and nutrient medium. With this object in view we performed experiments of the kind indicated in Table VII in which the trypanocidal titre of a single human serum was tested in six different nutrient media, each consisting of equal parts of the deactivated serum of a sheep and of Ringer-glucose solution.

It is seen from Table VII that the trypanocidal power of the same human serum manifested itself in widely different degree, according to the sheep serum with which it was diluted. For example, in the nutrient medium made with the serum of Sheep A human serum exhibited comparatively slight trypanocidal action even when present in concentration of 1 : 50, whereas with the media made from Sheep D and Sheep F there was evidence of pronounced trypanocidal action within four hours in concentrations of only 1 : 5,000 ; the remaining media gave intermediate results.

This experiment apparently indicated that the sera of different sheep varied in so far as some of them inhibited the trypanocidal action of normal human serum, whilst others did not, or did so only in a very minor degree. This interpretation is supported by another experiment in which, instead of sheep serum, we used the sera of two rabbits as the diluting media.

TABLE VII.

Showing the trypanocidal action of a single human serum when diluted to various extents with six different nutrient media, each consisting of equal parts of Sheep serum (heated to 60° C.) and of Ringer-glucose solution.

Human serum	Sheep serum	Concentration of human serum in nutrient medium (equal parts of sheep serum and Ringer-glucose solution)	Number of living parasites (*T. rhodesiense*) per 256 squares of the haemocytometer scale				
			Start	2 hours	4 hours	8 hours	22 hours
M	A	1 : 50		36	39	26	19
		1 : 100		38	24	24	13
		1 : 500		51	59	46	35
		1 : 1000		...	54	55	49
		1 : 5000		...	48	47	48
		Control		42	...	44	59
	B	1 : 50		33*	12	3	...
		1 : 100		22	3	...	...
		1 : 500		52	41	29	15
		1 : 1000		...	53	24	21
		1 : 5000		...	43	29	36
		Control		41	...	65	53
	C	1 : 50		36	29	23	12
		1 : 100		38	26	22	9
		1 : 500		43	55	55	58
		1 : 1000		...	49	59	41
		1 : 5000		...	47	48	62
		Control	48	49	...	45	40
	D	1 : 50		42*	10	1	...
		1 : 100		7	2	...	...
		1 : 500		6	2	...	...
		1 : 1000		6	2	...	...
		1 : 5000		20	8*	2	...
		Control		50	...	63	55
	F	1 : 50		41	26	28	15
		1 : 100		36	33	25	21
		1 : 500		60	55	56	47
		1 : 1000		...	48	48	54
		1 : 5000		...	46	52	66
		Control		47	...	52	58
	F'	1 : 50		8	5	1	...
		1 : 100		7	1	...	...
		1 : 500		8	2	...	...
		1 : 1000		15	1	...	...
		1 : 5000		30	27	10*	0
		Control		49	...	46	54

* Dead and disintegrating trypanosomes seen.

EXPERIMENT. Blood was obtained from the heart of each of two Rabbits A and B. It was allowed to clot for two hours at 37° C. and the serum removed. The serum of each animal was then divided into five portions which were dealt with as follows :—

     Portion I, unheated.

     Portions II to V, heated to 56° C. for 20 minutes.

All the specimens of serum were then stored overnight in the ice chest ; and the following morning,—

     Portion III, heated to 56° C. for 20 minutes.

     Portion IV, heated to 60° C. for 20 minutes.

     Portion V, heated to 64° C. for 20 minutes.

All the specimens of serum were then diluted with equal volumes of Ringer-glucose solution, and the mixtures were used in the usual way for the determination of the trypanocidal titre of the serum of a normal human being, as is shown in Table VIII.

The results set forth in Table VIII show clearly the influence exerted by the serum of different rabbits. With the serum of Rabbit A as a diluent, we find that the trypanocidal power of a certain normal human serum was, in five hours, manifest only in dilutions up to 1 : 100, whereas with the serum of Rabbit B it was, in the same period, manifest up to dilutions of between 1 : 1,000 and 1 : 10,000. The heating of the rabbit sera to the various degrees shown in the table exercised no effect on the result.

The most reasonable explanation of these results appears to be that the sera of certain sheep or rabbits have an inhibitory action on the trypanocidal power of human serum. This hypothesis receives further support from the following observation. The mixture of the sera of two different sheep—one of which allows of the manifestation of the trypanocidal action of human serum in great dilution, and the other only in comparatively low dilution—invariably gives results corresponding to the latter, i.e. apparently slight trypanocidal action.

Having reached the position that the sera of certain sheep and of certain rabbits possess the power of inhibiting or neutralising the trypanocidal property of human serum, whereas the sera of other sheep or rabbits do not possess this power—or if so only in an insignificant degree—we spent some time endeavouring to discover whether heating the animal serum to various temperatures, or storing it for various lengths of time, in any way affected its anti-trypanocidal properties. As is clearly shown in Table VIII, heating rabbit serum to various extents up to 64° C. in no wise altered its

TABLE VIII.

Showing the trypanocidal action of a single human serum when diluted to various extents with two different nutrient media, each consisting of equal parts of the serum of a rabbit and of Ringer-glucose solution.

Rabbit	Constitution of diluting medium consisting of equal parts of rabbit serum, heated or unheated, and of Ringer-glucose solution	Concentration of human serum	Number of living trypanosomes (T. rhodesiense) per 256 squares of the haemocytometer scale					
			Start	2 hours	3 hours	5 hours	8 hours	20 hours
A	Unheated rabbit serum	1:1000		34	...	38	...	36
	Rabbit serum heated once to 56° C.	1:10		24*	5*	0	...	...
		1:100		13*	2	0	...	...
		1:1000		34	...	39	14	1
		1:10000		...	42	32	56	45
		Control		...	...	55	54	51
	Rabbit serum heated twice to 56° C.	1:10		25*	18*	1	...	...
		1:100		11*	3	2	...	...
		1:1000		32	...	32	26*	8
		1:10000		...	40	40	53	46
		Control		...	...	47	49	31
	Rabbit serum heated once to 56° C. and once to 60° C.	1:10		8*	3*	0	...	...
		1:100		8	2	2	...	...
		1:1000		...	45	47	41	35
		1:10000		...	42	45	49	31
		Control		...	...	48	52	41
	Rabbit serum heated once to 56° C. and once to 64° C.	1:10		22*	7	2	...	...
		1:100		7	0	...	...	...
		1:1000		...	40	35	38	26
		1:10000		...	33	51	42	21
		Control		...	...	44	47	18
B	Unheated rabbit serum	1:1000	36	8	...	...	0	...
	Rabbit serum heated once to 56° C.	1:10		0	...	...	...	...
		1:100		1	1	0	...	...
		1:1000		2	3	2	...	...
		1:10000		...	40	25	17*	...
		Control		...	...	42	49	34
	Rabbit serum heated twice to 56° C.	1:10		4	0	...	...	...
		1:100		3	0	...	...	...
		1:1000		5	5	0	...	...
		1:10000		...	44	20	11*	9
		Control		...	...	54	56	49
	Rabbit serum heated once to 56° C. and once to 60° C.	1:10		10*	1	0	...	...
		1:100		1	1	0	...	...
		1:1000		12	9	3	...	...
		1:10000		...	16	18	13*	0
		Control		...	46	45	48	21
	Rabbit serum heated once to 56° C. and once to 64° C.	1:10		2	3	0	...	...
		1:100		8	7	3	...	...
		1:1000		16	9	3	...	...
		1:10000		...	24	24	3*	...
		Control		...	47	45	44	22
	Unheated human serum	Pure		...	0	...	...	...

' Dead and disintegrating forms seen.

properties in this respect. On the contrary, we have repeatedly found that rabbit or sheep serum, which has been stored for four or five days, exhibited a greater anti-trypanocidal action than did the freshly-obtained deactivated serum.

These questions are, however, beyond the scope of our immediate aim, which is to devise a method for determining the trypanocidal titre of normal human serum. With this object in view we selected a sheep, the serum of which was found on one occasion to exhibit no anti-trypanocidal action, and decided with the aid of this serum to examine the trypanocidal titres of a number of normal human sera on various occasions in order to ascertain whether constant results were obtained. This, in fact, proved to be the case as is shown in Table IX, from which it will be seen that when equal parts of the serum of this sheep and of Ringer-glucose solution were used as the nutrient and diluent medium, practically constant results were obtained with a number of human sera on various occasions.

From this work it appears that with a trypanosome concentration of about 1,000 per cmm., normal human serum possesses sufficient trypanocidal power to destroy *in vitro* at 37° C. all the parasites within a period of four hours when it is present in concentration of 1 : 5,000 ; within a period of eight hours in concentration of 1 : 10,000 ; and within eighteen hours in concentration of 1 : 25,000, in the case of *T. rhodiesense* : and in concentration of about 1 : 500 in four hours, of 1 : 1,000 in eight hours, and of 1 : 2,500 to 1 : 5,000 in eighteen hours, in the case of *T. equiperdum.*

These figures imply that 1/10,000 c.c. of human serum can destroy within eight hours all the parasites in 1 c.c. of a suspension containing 1,000 *T. rhodesiense* per cmm., or, in other words, one million trypanosomes. From this it follows that 1 c.c. of human serum contains sufficient trypanocidal substance to destroy *in vitro* at 37° C. 10,000 million trypanosomes.

When we approached from the other direction the question of the titre of the trypanocidal power of human serum, viz., by adding different numbers of trypanosomes to a constant concentration of human serum, the difficulty which confronted us was that of discovering a medium in which great numbers of trypanosomes could be kept alive at 37° C. for the length of time necessary to judge of the trypanocidal action of human serum.

TABLE IX

Summarizing the observations made on the trypanocidal titre of the serum and plasma of various normal human beings; the diluent consisted of equal parts of a selected sheep serum (heated to 60° C.) and of Ringer-glucose solution

Individual	Date	Serum or plasma	Trypanosome	Minimum concentration of serum or plasma which killed at least 95% of the parasites.		
				4 hours	8 hours	18 hours
A.R.D.A.	19.12.29	Serum	*T. rhodesiense*	1 : 5,000	1 : 10,000	...
A.Y. ...	24.1.30	,,	,,	1 : 10,000	...	...
A.C. ...	4.2.30	,,	,,	1 : 2,500	1 : 10,000	1 : 25,000
D.D. ...	24.1.30	,,	,,	1 : 5,000	1 : 10,000	...
F.S. ...	24.1.30	,,	,,	1 : 10,000	...	...
A.F.G. ...	24.1.30	,,	,,	1 : 5,000	1 : 10,000	...
J.R. ...	24.1.30	,,	,,	1 : 2,500	1 : 5,000	1 · 10,000
F.M. ...	14.1.30	,,	,,	1 : 5,000	...	1 : 25,000
,, ...	14.1.30	Plasma	,,	1 : 5,000	...	1 : 25,000
J.M. ...	20.1.30	Serum	,,	1 : 5,000	...	...
T.S. ...	4.2.30	,,	,,	1 : 2,500	1 : 5,000	1 : 10,000
W.Y. ...	20.1.30	,,	,,	1 : 5,000	...	...
,, ...	24.1.30	,,	,,	1 : 5,000	1 : 10,000	...
A.R.D.A.	20.12.29	Serum	*T. equiperdum*	1 : 500	1 : 1,000	...
,, ...	20.12.29	Plasma	,,	1 : 500	1 : 1,000	...
,, ...	28.1.30	Serum	,,	1 : 500	1 : 1,000 (partial)	1 : 5,000 (almost)
A.C. ...	4.2.30	,,	,,	1 : 500 (partial)	1 : 500	1 : 2,500 (almost)
T.S. ...	4.2.30	,,	,,	1 : 100 (partial)	1 : 500	1 : 2,500 (almost)

'Partial' implies that about 50 per cent. of the parasites were destroyed, and 'almost' over 75 per cent.

In our previous paper (1929) we have referred to this matter in considerable detail, and have shown that the addition of glucose to sheep or rabbit serum, and the frequent agitation of the suspension, sufficed to add considerably to the length of life of the trypanosomes.

EXPERIMENT. Normal sheep serum and normal human serum were diluted with equal parts of Ringer-glucose solution. To one tube of each of the diluted sera was added 1/20th vol. of a trypanosome suspension of such a strength that the final concentration of parasites was about 40 per 256 squares of the haemocytometer scale (625 trypanosomes per cmm.); and to another tube of each was added 1/20th vol. of a trypanosome suspension twenty times as strong, so that the final concentration of parasites was 800 per 256 squares of the haemocytometer scale (12,500 trypanosomes per cmm.). The tubes were then placed in a water-bath at 37° C. and the contents shaken from time to time. The results of examination after various periods are shown in Table X.

TABLE X.

Showing the trypanocidal action *in vitro* at 37° C. of a constant volume of human serum on different concentrations of trypanosomes

Tube		Number of living trypanosomes (*T. rhodesiense*) per 256 squares of the haemocytometer scale.				
		Start	1¾ hours	3 hours	4 hours	16 hours
1	Human serum 0·25 c.c. + Ringer-glucose sol. 0·25 c.c.	40 {	2	0	...	...
2	Sheep serum 0·25 c.c. + „ „		40	...	47	38
3	Human serum 0·25 c.c. + „ „	[800] {	++*	34*	11*	0
4	Sheep serum 0·25 c.c. + „ „		+++	+++	+++	250

The figure in brackets is a calculated number.
+++ parasites apparently all healthy and too numerous to count.
* Dead and disintegrating forms seen.

From Table X it is seen that even when the concentration of trypanosomes was twenty times that usually employed, the great majority of the parasites was destroyed by the human serum within an hour or two, whilst even after sixteen hours large numbers were still alive in the sheep serum. Hence it appears that 1 c.c. of human serum contains sufficient trypanocidal substance to destroy, within three hours, at least 25 million trypanosomes.*

* The concentration of trypanosomes in the ·5 c.c. of suspension (·25 c.c. human serum + ·25 c.c. Ringer-glucose) was 800 per 256 squares of the haemocytometer scale, i.e., 12,500 per cmm. or 6,250,000 per ·5 c.c. These were destroyed by ·25 c.c. of human serum and therefore 1 c.c. of human serum could destroy at least 25,000,000 trypanosomes.

Further experiments showed, however, that 1 c.c. of human serum contained sufficient trypanocidal substance to destroy infinitely greater numbers of parasites. In the experiment which we are about to describe advantage was taken of a fact, to which reference has already been made, viz., that we had been able to select a sheep, the serum of which exhibited no anti-trypanocidal action. This discovery enabled us to employ the serum of this sheep at the same time both as a nutrient medium and as a suitable diluent for human serum.

EXPERIMENT. Glucose to the extent of 0·25 per cent. was added to deactivated sheep serum ; 1 c.c. of the glucose-serum was placed in each of Tubes 1-4, and to Tubes 2 and 4 was then added 0·01 c.c. of human serum. Two suspensions—one twelve times as strong as the other—of washed trypanosomes from a mouse heavily infected with *T. equiperdum* were then prepared. To Tubes 1 and 2, was added 0·05 c.c. of the weak trypanosome suspension, and to Tubes 3 and 4, 0·05 c.c. of the strong suspension. The final concentration of trypanosomes was in the case of the weaker 70 per 256 squares of the haemocytometer scale (1100 per cmm.), and in that of the stronger 840 per 256 squares (13,000 per cmm.). The tubes were placed in the water-bath at 37° C. and examined at various intervals with results shown in Table XI. As after 3½ hours practically all the trypanosomes in Tubes 2 and 4 containing 1 per cent. human serum were dead, Tubes 3 and 4 were centrifuged at high speed and the supernatant fluids removed and placed in Tubes 5 and 6 to each of which was then added 0·05 c.c. of an exceedingly strong suspension of *T. equiperdum*, the resulting concentration of parasites being 2,500 trypanosomes per 256 squares of the haemocytometer scale (40,000 per cmm.). Tubes 5 and 6 were then placed in the water-bath and observed from time to time as shown in Table XI.

From the figures shown in Table XI it appears that 0·01 c.c. of human serum destroyed all the trypanosomes contained in 1 c.c. of a suspension of 840 per 256 squares of the haemocytometer scale (13,000 per cmm.), and subsequently those in 1 c.c. of a suspension of 2,500 trypanosomes per 256 squares (40,000 per cmm.) ; in other words, 1 c.c. of human serum was able to destroy at least 5,300 million trypanosomes.

There seems no reason to doubt that the experiments recorded above have established beyond all question the fact that human serum and human plasma exert *in vitro* at 37° C. a powerful trypanocidal action on our old laboratory strains of *T. rhodesiense* and *T. equiperdum*.

On the two strains of *T. congolense*, viz., ' Strain H ' and ' Strain S,' the trypanocidal action of normal human serum was likewise quite definite ; it was easily demonstrable in dilutions up to 1 : 10, but beyond this point the action was

indefinite. As we have previously stated normal human serum had no apparent action *in vitro* on our old laboratory strain of *T. gambiense*.

The reaction of the ' Sherifuri K ' strain was peculiar. This strain had been isolated from man twelve months before it was examined, and during this period had been maintained by passage through a series of five guinea-pigs. As compared with our old laboratory strains of *T. rhodesiense* and *T. gambiense*, which had

TABLE XI.

Showing the trypanocidal action *in vitro* at 37° C. of a 1 per cent. solution of human serum in a sheep serum-glucose medium, on different concentrations of trypanosomes.

Tube		Number of living trypanosomes (*T. equiperdum*) per 256 squares of the haemocytometer scale.				
		Start	1 hour	2 hours	3½ hours	10 hours
1	Sheep serum-glucose 1 c.c. + ...	70	⌠68	69	74	66
2	„ „ „ + human serum 0·01 c.c.		⌡37*	6*	0	...
3	Sheep serum-glucose 1 c.c.	840	⌠800	880	790	...
4	„ „ „ + human serum 0·01 c.c.		⌡780	440*	1*	...

After 3½ hours Tubes 3 and 4 centrifuged and the supernatant fluids transferred to Tubes 5 and 6; to each was then added 1/20th vol. of an exceedingly strong suspension of *T. equiperdum*.

		Start	4 hours	6 hours		
5	Supernatant fluid of Tube 3	2,500	⌠1,880	1,800	...	...
6	Supernatant fluid of Tube 4		⌡160*	150*	...	...

* Dead and disintegrating forms seen.

been maintained by passage through large numbers of mice for seven or eight years, it was therefore a comparatively young strain. It proved to be slightly sensitive to the trypanocidal action of human serum ; undiluted serum destroyed the parasite within four to eight hours, but serum diluted five- or ten-fold had no action. As regards its serum-resistance, the ' Sherifuri K ' strain thus

occupies a position intermediate between our old laboratory strains of *T. gambiense* and *T. rhodesiense* (Table XII). This appears to us to be a matter of great interest and we refer to the subject later in the present paper.

TABLE XII

Showing the trypanocidal action *in vitro* at 37° C. of three normal human sera on *T. rhodesiense*, *T. gambiense*, and 'Sherifuri K' strain, respectively.

Normal human serum	Concentration of human serum in nutrient medium (equal parts of selected sheep serum and Ringer-glucose solution).	Trypanosome	Number of living trypanosomes per 256 squares of the haemocytometer scale					
			Start	1 hour	2 hours	4 hours	8 hours	12 hours	18 hours
A.R.D.A.	Pure human ...	*T. rhodesiense*	33	0	...	...	...	...
	1 : 5 ...	"	38	9	0	...	...	...
	1 : 10	"	32	6	0	...	...	...
	1 : 50	"	39	9	0	...	...	...
	1 : 100	"	...	7	3	0	...	...
	1 : 500	"	...	18	1	0	...	...
	1 : 1000	"	...	24	6	3	...	...
	1 : 5000	"	...	50	26	5	...	...
W.Y.	Pure human ...	"	5	0	...	...	...	...
	1 : 5 ...	"	5	0	...	...	...	...
	1 : 10	"	26	2	0	...	...	...
	1 : 50	"	37	1	0	...	...	...
	1 : 100	"	...	3	0	...	...	...
	1 : 500	"	...	4	2	0	...	...
	1 : 1000	"	...	10	2	0	...	...
	1 : 5000	"	...	22	10	3	...	...
F.M.	Pure human ...	"	5	0	...	...	...	...
	1 : 5 ...	"	4	0	...	...	...	...
	1 : 10	"	12	0	...	...	...	...
	1 : 50	"	11	0	...	...	...	...
	1 : 100	"	...	0	...	...	...	...
	1 : 500	"	...	3	1	0	...	...
	1 : 1000	"	...	7	1	0	...	...
	1 : 5000	"	...	29	9	2	...	...
Control ...	Sheep serum + Ringer-glucose	"	60	66	64	61	49	51
A.R.D.A.	Pure human ...	*T. gambiense*	58	59	63	53	52	35
	1 : 5 ...	"	61	65	53	47	58	...
	1 : 10	"	...	...	...	58	...	...
	1 : 50	"	...	...	67	...	...	...
	1 : 100	"	...	...	...	...	...	...
W.Y.	Pure human ...	"	63	55	52	51	35	15
	1 : 5 ...	"	64	64	...	63	59	...
	1 : 10	"	...	...	61	...	...	...
	1 : 50	"	...	...	...	...	...	...
	1 : 100	"	...	...	...	...	...	...
F.M.	Pure human ...	"	57	54	65	57	46	50
	1 : 5 ...	"	62	61	...	54	56	...
	1 : 10	"	...	...	...	55	...	...
	1 : 50	"	...	...	...	...	...	...
	1 : 100	"	...	...	63	...	...	...
Control ...	Sheep serum + Ringer-glucose	"	57	58	58	63	41	20

Note: The "Start" column shows a bracketed value of 60 spanning the *T. rhodesiense* rows and 56 spanning the *T. gambiense* rows.

TABLE XII—*Continued.*

Normal human serum	Concentration of human serum in nutrient medium (equal parts of selected sheep serum and Ringer-glucose solution).	Trypanosome	Number of living trypanosomes per 256 squares of the haemocytometer scale						
			Start	1 hour	2 hours	4 hours	8 hours	12 hours	18 hours
A.R.D.A.	Pure human	'Sherifuri K'	22	24	24	1	0	...	
	1 : 5	„		...	72	66	49	52	47
	1 : 10	„		...	...	67	56	64	57
	1 : 50	„		...	...	...	78	65	63
	1 : 100	„		...	...	...	...	...	...
	1 : 500	„		...	...	61	...	...	...
	1 : 1000	„		...	...	...	...	...	...
	1 : 5000	„		...	...	72	...	...	...
W.Y.	Pure human	„		49	48	42	3	0	...
	1 : 5	„		...	...	55	65	58	54
	1 : 10	„		...	...	64	70	61	61
	1 : 50	„	75	...	...	...	...	49	...
	1 : 100	„		...	...	61	...	55	...
	1 : 500	„		...	...	...	...	58	...
	1 : 1000	„		...	...	...	...	61	...
	1 : 5000	„		...	...	61	...	57	...
F.M.	Pure human	„		13	17	2	0	...	...
	1 : 5	„		...	...	52	19	7	2
	1 : 10	„		...	...	63	21	5	5
	1 : 50	„		...	...	73	47	42	38
	1 : 100	„		...	...	...	51	54	53
	1 : 500	„		...	...	...	65	61	54
	1 : 1000	„		...	...	78	...	63	52
	1 : 5000	„		...	...	...	...	...	56
Control ...	Sheep serum + Ringer-glucose ...	„	78	...	72	70	72	62	

DISCUSSION

We must now pass to a discussion of the significance of the trypanocidal properties of human serum, and consider whether this remarkable quality is related to the equally striking relative immunity of man to infection with the trypanosomes pathogenic to the domesticated animals, and indeed to all members of the genus TRYPANOSOMA, except *T. gambiense*. The problem is undoubtedly a fundamental one and its correct solution would in all probability throw much light on the subject of the epidemiology of human trypanosomiasis, and particularly on that variety due to *T. rhodesiense :* it might even go far towards clarifying the important and still vexed questions of the identity of *T. brucei* and *T. rhodesiense*,

and the antelope reservoir of the latter parasite ; and of the relationship of these parasites with *T. gambiense*.

As we have already pointed out in the historical introduction to this paper, when Laveran and Mesnil first discovered the fact that human serum had a definite therapeutic effect on mice infected with *T. brucei*, they were inclined to associate the phenomenon with man's immunity to this parasite. The fact, however, that Laveran and Mesnil, and many others who followed them, were unable to demonstrate that human serum had any action *in vitro* tended to shake confidence in this view, as also did Laveran and Mesnil's statement that human plasma was much less active than the serum. Rosenthal, who, judging from the number and magnitude of his publications on the therapeutic action of human serum in experimental trypanosomiasis, must have devoted a great amount of time to the subject during the last fifteen years, and whose views, therefore, merit special consideration, has, for a number of reasons which appear to him cogent, reached the definite conclusion that the therapeutic and protective property of human serum is in no way related to man's immunity to the pathogenic trypanosomes of animals.

The question is of such practical importance that it appears to us desirable to summarize once more the main observations upon which Rosenthal bases his views, and then to subject them to critical examination. Rosenthal's points, which are marshalled in a recent paper (1924), are as follows :—

1. The lower apes, sheep, goats and pigs are but slightly susceptible to trypanosomal infections, and hens and geese are completely insusceptible, and yet the serum of these animals contains no trypanocidal substance ; consequently, either the biological mechanism of natural resistance to the pathogenic trypanosomes must vary in the different classes of animals, thus rendering impossible a uniform explanation of trypanosome immunity, or the heart of the matter has not been laid bare by the discovery of the therapeutic action of human serum.

2. Laveran and Mesnil have shown that the trypanocidal action of human serum is, in considerable measure, a coagulation effect, because human plasma exhibits but little therapeutic action as compared with serum. From this it appears that either anti-trypanocidal substances are present in human plasma which are eliminated in the process of coagulation, and do not reach the necessary degree of inhibitory action in the human body, or the trypanocidal power of human serum is in great part an artificial product and is not present in the circulating blood.

3. The most weighty observation is, however, that human serum has no direct lethal action on trypanosomes, and that its trypanocidal action first becomes manifest after its incorporation into the organism of a foreign species of animal; trypanosomes

can be left for several hours in contact with human serum without visible injury, and if such parasites be freed from the serum by centrifuging and washing, they produce infection when injected into a susceptible animal. In other words, Rosenthal believes that there exists in human serum a trypanocidogenous substance which, after injection into an animal, is converted by the reticulo-endothelium of that animal into trypanocidal substance. The fact that the trypanocidal substance is not present in a pre-formed state in human serum shows on what shifting ground the teaching of serum-trypanocidicity as the foundation of man's immunity to the pathogenic trypanosomes of animals rests.

The first of these three arguments does not appear to us to have much weight. The lower apes, sheep, goats and pigs are all susceptible to infection with the trypanosomes pathogenic to man and stock. It is probably true that in certain native herds of sheep, goats and pigs, the infections may be exceedingly mild, and that the animals may exhibit a tolerance to infection almost comparable to that exhibited in so remarkable a degree by the antelope, but they can in no sense be said to be immune to infection with any of the trypanosomes pathogenic to man and stock. It is, furthermore, hardly correct to state that fowls and geese are completely insusceptible to trypanosomal infections. It is true that the Royal Society's Commission in Uganda (1911) was unable to infect fowls with *T. gambiense* by means of *G. palpalis* : but there is considerable evidence that at least a temporary infection of both fowls and geese can follow inoculation of infected blood. Schilling (1904), and Mesnil and Martin (1906) infected geese with nagana, but failed to infect fowls. Goebel (1906 and 1908), however, inoculated *T. brucei* into the caruncles of fowls ; twenty-three birds were used in these experiments and although trypanosomes were never found in the circulating blood, or in smears of organs, Goebel obtained infections in guinea-pigs sub-inoculated with the blood of the fowls in twenty cases, over periods varying from three to eighty-one days. It was observed that passage through the fowl did not alter the virulence of the trypanosome for guinea-pigs ; as a rule, the fowls exhibited no appreciable symptoms.

Mesnil and Blanchard (1912) found that fowls could be infected with *T. gambiense* and *T. rhodesiense :* four were inoculated with each trypanosome, 1 c.c. of diluted mouse-blood being injected into the wattles, and seven of the eight became infected. Trypanosomes were not seen in blood films, but the blood was shown to be infected by sub-inoculation into rats every ten or twelve days. Three of the

fowls infected with *T. gambiense* died on the 28th, 38th and 75th days, respectively, and two of those inoculated with *T. rhodesiense* on the 56th and 62nd days. Four of the fowls had ocular lesions— conjunctivitis, iridocyclitis and keratitis—identical with those found in infected rabbits and dogs. We thus see that there is overwhelming evidence that fowls and geese are by no means immune to the pathogenic trypanosomes of man and stock.

Let us now turn to Rosenthal's second point, that the trypanocidal action of human serum is in considerable measure a coagulation effect because human plasma exhibits but little therapeutic action as compared with serum. So far as we have been able to ascertain, this statement is based not upon Rosenthal's personal observation, but upon one made by Laveran and Mesnil ; these authors (1902) write : ' Dans une expérience, le plasma humain citraté s'est montré très peu actif, en comparaison du sérum.'

It is interesting to note that a deduction of such importance as to justify frequent citation in the literature is apparently based on a single experiment which, so far as we can discover, has never been confirmed. We shall, however, leave this subject for the moment and pass to a consideration of Rosenthal's third and most important argument, viz., that the power of human serum to cure infected mice is not related to man's immunity to the pathogenic trypanosomes of animals, because human serum has no lethal action on the trypanosomes *in vitro*, but only becomes trypanocidal after its incorporation into the organism of the injected animal.

This would, of course, be an almost overwhelming argument if it were a fact, but our experimental work has convinced us that it is most definitely not so. We have demonstrated conclusively that normal human serum exerts a pronounced trypanocidal action on our old laboratory strains of *T. rhodesiense* and *T. equiperdum in vitro* at 37° C., and that this trypanocidal action is manifest even when the trypanosomes are present in enormous concentration, or, alternatively, when the serum is enormously diluted. The trypanocidal action of human serum on our two strains of *T. congolense* was, although much less pronounced, still quite definite, whilst with our old strain of *T. gambiense* we were unable to satisfy ourselves that human serum had any trypanocidal action *in vitro* ; the ' Sherifuri K ' strain proved to be partially serum-resistant.

A further important point brought out in our experimental work is that citrated human plasma exhibits *in vitro* a trypanocidal action equal to that of the serum.

These facts seem to us to dispose once and for all of Rosenthal's two main arguments. We do not propose here to embark on an analysis and criticism of Rosenthal's experimental work on the therapeutic action of human serum in experimentally infected mice, much of which—particularly that relating to the part played by the reticulo-endothelium of the mouse—appears to be deeply interesting, but merely to content ourselves with recording that the results of our work, on the trypanocidal action of human serum *in vitro*, would afford adequate *prima facie* grounds for believing that the therapeutic effect is explicable on the hypothesis that the injected serum acts directly on the parasite without undergoing any change in the mouse organism. These are, however, matters which we must leave for a further paper.

The unequivocal demonstration that *T. rhodesiense, T. equiperdum* and *T. congolense* are rapidly destroyed *in vitro* at 37° C. by normal human serum or plasma, and that *T. gambiense* is apparently unharmed by either, endows the well-known phenomenon of the therapeutic action of human serum in experimentally infected mice with an entirely new significance, and seems to us to afford the strongest grounds for believing that man's immunity to infection with the pathogenic trypanosomes of stock, and his relative susceptibility to *T. gambiense*, are bound up with this property of his serum ; and that the phenomenon has, in all probability, an important bearing on the epidemiology of human trypanosomiasis.

This naturally brings us to the question why one parasite derived from man, viz., *Trypanosoma rhodesiense*, should be very susceptible to the trypanocidal property of normal human serum or plasma, whilst the other, viz., *Trypanosoma gambiense*, is exceedingly resistant. As we have already mentioned, our *T. rhodesiense* strain was isolated from man in 1923, and the *T. gambiense* strain in 1922, and both have since been maintained by passage through mice.

Unfortunately, we have no direct knowledge of the trypanocidal action *in vitro* of human serum on other strains of trypanosomes

derived from man* with the single exception of the very interesting ' Sherifuri ' strain to which we refer later.

The information supplied by Razgha (1929), in his recent paper on the cultivation of the trypanosomes pathogenic to man, may, however, not be without significance in this respect. Razgha employed as a culture medium equal parts of Ringer's solution and of citrated human blood ; it was found preferable to allow the medium to stand for several days before inoculation ; the cultures were incubated at 22°-24° C. The author records that in his experiments he used three strains of *T. gambiense*—one eight years old and two recently isolated from man ; and two strains of *T. rhodesiense*—five and seventeen years old, respectively. With the freshly isolated strains of *T. gambiense*, cultures were obtained without difficulty, but only occasionally with the older strain of *T. gambiense* and the younger strain of *T. rhodesiense*, and never with the older strain of *T. rhodesiense*. Attempts to cultivate the laboratory strain of *T. brucei* were also unsuccessful.

In our opinion the most probable explanation of these divergent results is that the most recent *T. gambiense* strains were completely serum-resistant, that the older strain of *T. gambiense* and that the younger strain of *T. rhodesiense* were partially resistant, whilst the older strain of *T. rhodesiense* and the laboratory strain of *T. brucei* were very sensitive to the trypanocidal action of human serum.

Furthermore, there is in the literature a number of observations relating to the prophylactic and therapeutic action of normal human serum on mice infected with *T. gambiense* and *T. rhodesiense*. Laveran (1904), Thiroux and d'Anfreville (1908), and Mesnil and Leboeuf (1910), all showed that human serum exercised no action on the course of *T. gambiense* infections in mice ; whilst Mesnil and Ringenbach (1911) made the interesting discovery that *T. rhodesiense* in mice was very susceptible to the action of human serum.

Some years later, Mesnil and Blanchard (1916) summarized the available information regarding the sensitiveness to human serum of several strains of trypanosomes carefully observed from the time of their isolation from man. They state that two strains of

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* Saito (1927), in the German summary of his Japanese paper, states that human serum exerted *in vitro* a definite trypanocidal action on a strain of *T. gambiense* which had been preserved for many years by passage through mice.

T. rhodesiense proved to be relatively sensitive to human serum shortly after they were obtained from man. We have traced these two strains back through the literature and find that the first was isolated from the original *T. rhodesiense* case, W.A., by one of us, in December, 1909, and sent to Mesnil ; this strain then at the time it was tested by Mesnil and Ringenbach (1911) was probably about eighteen months old. The other strain was sent to Mesnil by Wenyon, who isolated it in 1911, or 1912, from a case in London, W.G., so that at the time it was first tested by Mesnil and Ringenbach, in 1914, it was approximately two years old. As regards *T. gambiense*, one strain studied by Laveran (1915) proved to be insusceptible to the action of human serum twelve years after it was isolated from man. The second strain of *T. gambiense* (Mesnil's) first exhibited some slight degree of sensitiveness seven years after it was obtained from man (Mesnil and Ringenbach, 1912) ; afterwards its sensitiveness varied somewhat, but eventually after eleven years passage through laboratory animals, it became almost as sensitive to human serum as *T. rhodesiense* (Mesnil and Blanchard, 1916). It is further stated that the strain *lanfranchii*—the nature of which is uncertain, but which was obtained from an accidental laboratory infection of Professor Lanfranchi—was found to be susceptible to the action of human serum when it was first examined two years after its isolation from man.

Zeiss (1920) studied the therapeutic action of normal human serum in mice infected with four strains of *T. gambiense* and one strain of *T. rhodesiense*. Two of the *gambiense* strains had been preserved in laboratory animals for fifteen years and the other two for six years ; the *rhodesiense* strain had been obtained from man seven years previously. Human serum was found to have no therapeutic action on any of the *gambiense* strains, but to have a pronounced therapeutic action on the *rhodesiense* strain.

If we are correct in our assumption that the therapeutic action of human serum is merely an index of its actual trypanocidal properties—and we believe that our experiments have shown that this assumption is well-founded—then we can infer from our own observations, and from those of the workers just quoted, that whilst *T. rhodesiense* is very susceptible to the trypanocidal action of human serum within about one and a half to two years after its

isolation from man, *T. gambiense* remains insusceptible for prolonged periods, viz., our own strain for at least eight years, Laveran's strain for at least twelve years, Zeiss's four strains for at least six to fifteen years, whilst Mesnil and Ringenbach's strain remained insensitive for seven years, and then gradually during the following four years became susceptible.

The resistance of *T. gambiense* to the cytolytic action of human serum thus appears to be a remarkably fixed character, and it is difficult to avoid the conclusion that man's susceptibility to infection by *T. gambiense* is directly due to this fact. When, however, we turn to *T. rhodesiense*, the problem is not so simple. Here we have to deal with a parasite which within two years of its isolation from man is very susceptible to the cytolytic action of human serum. It seems to us incredible that our laboratory strain of *T. rhodesiense* which, as we have shown is killed by normal human serum, or plasma, *in vitro* at 37° C. within a few hours, even though the serum or plasma is diluted to such an extent that its concentration in the medium is only 1 : 25,000, could infect man ; and we are, therefore, compelled to conclude that it has changed radically since its isolation from man, and that *T. rhodesiense*, unlike *T. gambiense*, rapidly loses its serum-resistance when maintained in laboratory animals. In other words, the serum-resistance of *T. rhodesiense* is not a fixed or stable character, but is one which is relatively readily acquired and quickly lost.

In this connection it is interesting to consider the behaviour of the ' Sherifuri K ' strain. This strain is peculiar in that, although it was isolated from the gland juice of an infected native in a district in which so far as is known all human trypanosomiasis is of the *gambiense* type, it presented certain marked differences from the usual strains of *T. gambiense* isolated from man. Firstly, the sub-inoculated animals of the early passages exhibited numerous posterior-nuclear forms ; secondly, the pathogenicity in guinea-pigs appears to us to resemble much more closely that of the *T. rhodesiense* strains isolated from *rhodesiense* sleeping sickness cases by Kinghorn and Yorke (1912) in the Luangwa Valley, than that of recently isolated *gambiense* strains ; and thirdly, in an unpublished communication to Dr. Wenyon, it is stated that Paisley found that the adhesion test showed the parasite to be more closely related to the local strain of *T. brucei* than to that

of *T. gambiense*. It is further recorded that *G. morsitans* and *G. tachinoides* are present in the district from which the patient came, and that there is plenty of *T. brucei* in the game [Letter from Lloyd, 3rd March, 1930]. These facts, considered in conjunction with the remarkable behaviour of the parasite to normal human serum—it was partially serum-resistant, infinitely more so than our laboratory strain of *T. rhodesiense*, but definitely less so than our laboratory strain of *T. gambiense*—suggest to us that the ' Sherifuri K ' strain is *T. rhodesiense*, which at the time it was isolated from man in February, 1929, was serum-resistant, and during its passage through five guinea-pigs over a period of twelve months, has become slightly serum-sensitive. We hope to keep the ' Sherifuri K ' strain under careful observation and to examine its reaction to normal human serum from time to time.

These facts appear to us to throw interesting light on the difficult and important problem of the epidemiology of sleeping sickness due to *T. rhodesiense*. It will be recalled that Kinghorn and Yorke (1912 and 1913) discovered the important fact that the game, and particularly the antelope, of Central Africa, were widely infected with the trypanosomes pathogenic to the domesticated animals ; one of the parasites found by them in game was shown to be indistinguishable from *T. rhodesiense* found in man. These discoveries caused Kinghorn and Yorke to state that the game constituted the chief reservoir from which *G. morsitans* derived the trypanosomes pathogenic to man and stock. These observations were quickly confirmed by Bruce and his colleagues (1913), and the question of the identity or non-identity of the trypanosomes of the same morphological appearance occurring in game and man was soon the subject of a violent polemic. Taute (1913) allowed two considerable batches of laboratory-bred *G. morsitans* which had been infected with the antelope strain of *T. brucei* to feed on himself, with negative results. In a further experiment, he inoculated himself with 2 c.c. of the blood of a naturally-infected dog, and again the result was negative. Some years later, Taute and Huber (1919) recorded that they injected themselves and 129 natives with *T. brucei* from naturally-infected animals, without obtaining infection in a single instance. Taute and Huber consequently argued, and with considerable force, that the trypanosome found in game and stock

is different from that found in man or, in other words, that *T. brucei* and *T. rhodesiense* are different species and that the game is not the reservoir of the human parasite ; and in this they were supported by Kleine (1914) and many others. Yorke and Blacklock (1914), and Yorke (1920) rejoined that although it had not been experimentally demonstrated that man could be infected with the game trypanosome, it had, on the contrary, been shown that stock and game could be infected with the trypanosome from man, and that as Glossina could readily be infected from these animals, it was difficult to understand how the parasites of man could fail to be widely disseminated amongst the game. They referred to the curiously sporadic distribution of *rhodesiense* infection in man and to the rarity, and limited nature, of epidemics of this form of sleeping sickness, and finally explained Taute's and Taute and Huber's failures to infect man on the hypothesis that man was exceedingly resistant to all members of the genus TRYPANOSOMA, except *T. gambiense.*

We find support for this last contention of Yorke and Blacklock in our present experimental work. The pronounced sensitiveness to normal human serum of *T. rhodesiense* which has sojourned for some time—how long we do not yet know, but probably it will be found to be comparatively short—in vertebrate hosts other than man, provides almost overwhelming evidence that normal human beings cannot become infected with such strains, and supplies a simple explanation of the result obtained in Taute's and in Taute and Huber's experiments.

When, however, we turn to a consideration of the case of persons in an abnormal state of health, the position is not so simple. We have already, in the introductory portion of this paper, referred at considerable length to the numerous observations which have been made, showing that the serum of individuals suffering from various diseases, particularly those of the liver, fails to protect mice against infection with pathogenic trypanosomes, and has little or no therapeutic action. So far, we ourselves have not yet had time to examine this important matter to any great extent, but a few observations which we have made have shown that in certain pathological conditions, e.g., amoebic abscess of the liver, and obstructive jaundice, the serum may lose entirely its power to destroy trypanosomes

in vitro. There seems no obvious reason why such persons whose serum, from some cause or other, has lost its power of destroying trypanosomes, should not be susceptible to infection. Much further work will be required to ascertain exactly under what circumstances the trypanocidal power of the serum is lost, but the point seems to be well worth investigation as a step in the direction of obtaining some knowledge of the epidemiology of *rhodesiense* infections in man. Investigations on this matter can well be carried on in European laboratories, but as other and equally important researches can only be undertaken in Tropical Africa, we have decided, with a view to stimulating and guiding what we believe to be important work, to conclude this paper by stating briefly the hypothesis we have elaborated regarding the epidemiology of *rhodesiense* and *gambiense* sleeping sickness, and by indicating the lines by which our hypothesis can be put to the test. The hypothesis is as follows :—

1. *T. rhodesiense* is synonymous with *T. brucei,* and the antelope and other game of Central Africa constitute, as was claimed by Kinghorn and Yorke, the reservoir.

2. Normal human beings cannot be infected with the parasite derived from game—either by blood injection or by the intermediary of Glossina—because of the remarkable trypanocidal power of human blood (plasma).

3. In individuals suffering from certain diseases, and probably also in those suffering from the effects of insufficient or improper dietary, the trypanocidal power of the blood is lost, and consequently such persons can be infected by Glossina which have drawn the virus from game.

4. When once the game trypanosome has established itself in an individual of this kind, it by degrees becomes serum-resistant— a process which is accelerated if, for any reason, the cause of the initial decrease or absence of trypanocidal substance is removed and the serum gradually recovers its lost power.

5. After it has sojourned in the human host for some time, the trypanosome is, like *T. gambiense,* definitely serum-fast, and consequently—unlike the parasite derived directly from game— infective for man.

6. The serum-fastness is possibly preserved during the passage of the parasite through Glossina ; and if so, the infection could spread

from man to man through cyclically infected tsetse. If, however, this proves not to be the case, then the only obvious explanation of such localised epidemics of this form of the disease as have occurred, e.g., the Mwanza epidemic, is either that the fly transmits mechanically the serum-fast parasite from man to man, or that some local condition exists, e.g., dietary deficiency, or hookworm disease, which so affects the population as to deprive many individuals of the protection due to the trypanocidal power of the blood.

7. If the parasite is passed on by tsetse to game or domestic animals instead of to man, it quickly loses its serum resistance—how quickly we do not yet know, but the available evidence suggests that the period is between one and two years—and so again becomes incapable of infecting normal man.

8. *T. gambiense* is, like *T. rhodesiense*, identical with *T. brucei*, its apparent greater differences being merely due to more profound modification resulting from numerous passages through the human host. In other words, the source of both trypanosomes pathogenic to man—*T. gambiense* and *T. rhodesiense*—is *T. brucei*, of which the natural reservoir is the game. The game trypanosome is not pathogenic to normal man because of the protective trypanocidal action of his blood. Under certain conditions (pathological and dietetic) the trypanocidal substance disappears from the blood of man and he becomes susceptible to infection with the game trypanosome. If such an individual becomes infected he develops a *rhodesiense* infection. These infections are seen typically in *morsitans* regions where the chief food supply of the fly is game or stock ; they are of sporadic occurrence and are unlikely to assume the form of considerable epidemics. We believe that *gambiense* infections likewise originated in this manner, but that having occurred in *palpalis* regions, where the contact between man and fly is more intimate than in *morsitans* regions, there was a correspondingly greater chance of man to man infection. Prolonged man-Glossina-man passage has produced the modifications of the parasite which have resulted in the characters of *T. gambiense*, the most striking of which is its ' fixed ' resistance to the trypanocidal action of human serum.

This hypothesis has, of course, one very serious disadvantage and that is that it rests on but a very slender foundation of experi-

mental observation. Nevertheless, as it appears to afford a reasonable explanation of the facts—morphological, biological and epidemiological—so far accumulated, it seems well worth subjecting to the test of experimental enquiry. This can, in our judgment, be done in a satisfactory manner by obtaining the answers to the following questions :—

1. Is *T. rhodesiense* in the blood of an infected man resistant to the trypanocidal action of normal human serum ? If not, is the serum of the patient without trypanocidal action on *T. brucei* obtained from game, stock, or wild tsetse ? Assuming—as seems highly probable—that *T. rhodesiense* in man is resistant to normal human serum, answers must be obtained to the following questions :—

2. Is *T. rhodesiense* in the blood of animals sub-inoculated from infected man resistant to the trypanocidal action of normal human serum ; and if so, for how long, and through how many animal passages is the serum-resistance maintained ?

3. Is the serum-resistant character preserved through the cyclical development of the trypanosome in Glossina ? This important question should easily be answered by infecting rats by Glossina which have been infected from man, and examining the serum-resistance of the parasite obtained from the blood of the rats. As a corollary to this investigation the effect of passing a serum-sensitive strain of *T. brucei* (or *T. rhodesiense*) obtained from game or stock through Glossina should be determined.

4. If the serum-resistant character is transmitted through Glossina, for how many Glossina-rat passages is the character maintained ?

5. Is there any difference in the serum-resistance of trypanosomes (*T. brucei* or *T. rhodesiense*) obtained from rats infected by Glossina caught respectively in regions where *rhodesiense* sleeping sickness is epidemic, and in regions where it is merely endemic or absent ?

6. Are the metacyclic trypanosomes from the salivary glands of Glossina infected, respectively, with *T. rhodesiense* from man, and *T. brucei* from game or stock, serum-resistant or serum-sensitive ?

7. Examine the trypanocidal power of the serum of the non-infected inhabitants of an area where *rhodesiense* sleeping sickness is endemic Does any considerable proportion of the population

exhibit a decrease or loss of serum trypanocidal power ? If so, is there any condition—dietetic or pathological—that can be held responsible ?

8. It is also possible that careful study of the reaction to the trypanocidal substance of normal human serum of the polymorphic trypanosome in the antelope, and wild *G. palpalis*, of the depopulated islands and littorals of Lake Victoria might furnish valuable information. Duke (1912 and 1921) has, in fact, produced some evidence, based mainly on studies of morphology and pathogenicity, which suggests that the polymorphic trypanosome from the situtunga antelope in Damba Island, had during the period 1912 to 1920, undergone certain changes. He writes : ' Whereas the 1912 strain agreed with *T. gambiense*, the 1920 strain shows many of the characters usually assigned to *T. brucei*.'

We have studied carefully Duke's papers, but are unable to convince ourselves that he succeeded in proving that the 1912 situtunga strain of Damba Island was undoubtedly *T. gambiense*. It is true that Duke produces some evidence that the 1920 antelope strain was more virulent for monkeys than was the 1912 antelope strain ; but we cannot but be impressed with the fact that the differences in pathogenicity between the 1912 and 1920 antelope strains appear, according to Duke's own data, to be distinctly less than those between the 1912 antelope strain and the strain of *T. gambiense* derived from man. The whole subject is one of very great difficulty and it is doubtful whether anything further is to be gained by analysing and discussing information relating to the 1912 strain. It does, however, seem to us to be a matter of importance to know what are the reactions to human serum of the present day antelope strain. Information on this subject might not only throw light on the nature of the antelope strain, but enable us to judge whether or not the trypanosome is capable of infecting man.

It seems to us that when answers have been obtained to these questions—and with the aid of the technique described in this and our previous papers, the task should be a comparatively simple one— we shall be in a position to examine critically the hypothesis set forth above and possibly to enjoy a much clearer insight into the important problem of the epidemiology of sleeping sickness than we do at present.

SUMMARY

1. The interesting discovery made by Laveran, in 1902, that normal human serum, when injected into mice suffering from nagana, exerted a marked effect on the course of the infection, has called forth an enormous number of papers dealing with different aspects of the subject.

2. In the historical survey which we have given of this work, it is pointed out that when Laveran and Mesnil first discovered the fact that human serum had a definite therapeutic effect on mice infected with *T. brucei*, they were inclined to associate the phenomenon with man's immunity to this parasite. The facts, however, that Laveran and Mesnil and many others who followed them were unable to demonstrate that human serum had any action *in vitro*, and that Laveran and Mesnil had found that plasma was but slightly active as compared with serum, tended to shake confidence in the view ; and Rosenthal, who has spent many years investigating the subject has reached the definite conclusion that the therapeutic and protective property of human serum is in no way related to man's immunity to the pathogenic trypanosomes of animals. Rosenthal believes that human serum contains a trypanocidogenous substance which, on injection into an animal, is converted into an active trypanocidal substance by the reticulo-endothelium of that animal.

3. In contrast to the experience of previous workers, we have succeeded in showing that normal human serum—and, equally, normal human citrated plasma—exerts a pronounced trypanocidal action *in vitro* at 37° C., on a number of strains of pathogenic trypanosomes. With our laboratory strains of *T. equiperdum* and *T. rhodesiense*, the trypanocidal action of normal human serum could be demonstrated even when the serum was diluted 5,000 and 25,000 times respectively ; with two recently isolated strains of *T. congolense*, the trypanocidal action was obtained with serum diluted up to ten times ;· with ' Sherifuri K ' strain—a recently isolated *rhodesiense*-like strain from a case of sleeping sickness in Northern Nigeria—the trypanocidal action was manifest with undiluted serum only ; but on our old laboratory strain of *T. gambiense*, normal human serum had no appreciable trypanocidal action.

4. The properties of the trypanocidal substance of normal human serum were studied, and a method described for determining its titre. In the course of this work the interesting fact was established that the sera of certain normal sheep and of certain normal rabbits possessed ' anti-trypanocidal' properties, in that when they were mixed with normal human serum, they prevented the trypanocidal action of the latter. The sera of other sheep and rabbits did not possess this property.

5. In certain pathological conditions, e.g., amoebic abscess of the liver, and obstructive jaundice, the serum loses its trypanocidal power, and exerts no cytolytic action on the trypanosomes *in vitro*.

6. The unequivocal demonstration that *T. rhodesiense*, *T. equiperdum* and *T. congolense* are rapidly destroyed *in vitro* at 37° C. by normal human serum or plasma, and that *T. gambiense* is apparently unharmed by either, endows the well-known phenomenon of the therapeutic action of human serum in experimentally infected mice with an entirely new significance, and appears to us to afford the strongest grounds for believing that man's immunity to infection with the pathogenic trypanosomes of stock, and his relative susceptibility to *T. gambiense*, are bound up with this property of his serum.

7. Evidence is adduced that resistance to the cytolytic action of human serum is a ' fixed ' character of *T. gambiense* which survives many years' passage through laboratory animals, but that the serum-resistance of *T. rhodesiense* is a ' labile ' character which is relatively quickly acquired and quickly lost. How long a strain of *T. rhodesiense* remains resistant to normal human serum after its isolation from man is not yet known, but we have produced some evidence to show that it becomes to some extent serum-sensitive within a year, after passage through a series of only five laboratory animals.

8. We believe that these facts have an important bearing on the epidemiology of human trypanosomiasis, and assist in clarifying the difficult and still vexed questions of the identity of *T. brucei* and *T. rhodesiense*, and the antelope reservoir of the latter parasite, and of the relationship of these parasites with *T. gambiense*.

9. We have elaborated a hypothesis which, in our view, affords a reasonable explanation of the facts, so far accumulated, regarding

the epidemiology of human trypanosomiasis in Africa. Briefly stated, the hypothesis is as follows :—*T. gambiense* is, like *T. rhodesiense*, identical with *T. brucei*, its apparent greater differences being merely due to more profound modification resulting from numerous passages through the human host. In other words, the source of both trypanosomes pathogenic to man—*T. gambiense* and *T. rhodesiense*—is *T. brucei*, of which the natural reservoir is the game. The game trypanosome is not pathogenic to normal man because of the protective trypanocidal action of his blood. Under certain conditions (pathological and dietetic) the trypanocidal substance disappears from the blood of man and he becomes susceptible to infection with the game trypanosome. If such an individual becomes infected he develops a *rhodesiense* infection These infections are seen typically in *morsitans* regions where the chief food supply of the fly is game or stock ; they are of sporadic occurrence and are unlikely to assume the form of considerable epidemics. We believe that *gambiense* infections likewise originated in this manner, but that having occurred in *palpalis* regions, where the contact between man and fly is more intimate than in *morsitans* regions, there was a correspondingly greater chance of man to man infection. Prolonged man-Glossina-man passage has produced the modifications of the parasite which have resulted in the characters of *T. gambiense*, the most striking of which is its ' fixed ' resistance to the trypanocidal action of human serum. As it is only in Tropical Africa that this hypothesis can be put to the test of experimental enquiry we have, for the guidance of those working in the field, indicated the manner in which this enquiry could most profitably be conducted.

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THE OCCURRENCE OF INTESTINAL PROTOZOAL INFECTIONS IN THE INHABITANTS OF LENINGRAD (U.S.S.R.) WITH SPECIAL REFERENCE TO *ENTAMOEBA HISTOLYTICA* CARRIERS

BY

A. A. PHILIPTSCHENKO, M.D.

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BACTERIOLOGICAL PASTEUR INSTITUTE IN LENINGRAD

The high rate of infections with intestinal protozoa in general and with *Entamoeba histolytica* in particular obtained for the healthy population of England, Sweden, the United States of America and other temperate countries, suggested the probability that the transmission of these parasites must also be widespread in the temperate regions of U.S.S.R. The few researches on this problem which were carried out with various aims, do not lead to a definite conclusion on this subject, because they were based on insufficient material and conducted on the basis of a method which has been found to be incorrect.

Such was the reason which compelled the Department of Parasitology of the Bacteriological Pasteur Institute in Leningrad, organized in May, 1929, to undertake the examination of various groups of inhabitants of Leningrad for intestinal protozoal infections and to consider this work as one of its principal problems.

The present report is the first of this series.

MATERIAL AND METHOD

An examination of 400 employees of the Workers' Co-operative Organisation of Leningrad and of 204 patients at one of the Leningrad dispensaries (the First Wiborg Polyclinic), was carried out during the period May to September, 1929.

In the first group were persons employed in various food-shops of Leningrad (Table XI below). In all these cases a careful inquiry

was made into the history of previous diseases and the present state of the gastro-intestinal system. The second group consisted of 162 adult workers and the third of 42 children from one to fourteen years old. All the adults suffered from various kinds of intestinal disorders, which were diagnosed as colitis cronica, enteritis, gastritis, enteroptosis, appendicitis and so on, but in the majority of cases as helminthiasis. The children also suffered from various intestinal disorders ; 32 cases were diagnosed as helminthiasis, and 10 cases as colitis haemorrhagica, enteritis, icterus, appendicitis and anaemia.

The stools were examined only once in each case. Two films, measuring 18 mm. by 36 mm., were prepared from the faeces (in the majority of cases twelve to twenty hours old) of every person. Before the films were allowed to dry, they were fixed with Schaudinn's fixative (with 5 per cent. of acetic acid) and immediately afterwards stained with Heidenhain's iron haemotoxylin. All the films were thoroughly and carefully examined at least twice, and often four times (the average time spent in examining each film was not less than ten minutes and in most instances as much as twenty minutes). The first examination was always made with the achromatic Zeiss 40 objective, and the second with the fluorite immersion Zeiss 100 objective and compensating eyepiece 7 ×. In some cases, in addition to examinations for cysts of protozoa, we also made examinations for the motile stage after a saline purgative had been administered. All the cysts of *E. histolytica* and *E. coli* were measured by a micrometric ocular and in each case the extreme sizes were noted.

The differentiation of cysts was based upon the following diagnostic features ; (1) size, (2) number and structure of the nuclei, and (3) morphology of the chromatoid bodies. All the cysts measuring from 6μ to 14μ, and containing one to four nuclei, corresponding to the type *Entamoeba*, having large bar-like oval, or spindle-shaped chromatoid bodies with rounded ends, were noted as *Entamoeba histolytica*. We did not make any differentiation between cysts of *E. tetragena, dispartenuis*, etc., bearing in mind the fact that, according to Dobell and others, they are only various races of *E. histolytica*. Cysts measuring from 12μ to 24μ with eight nuclei, were noted as *E. coli*. In the

majority of cases they did not show any chromatoid bodies, but in a few cases contained long, slender acicular bodies with fractured or sharp ends or filamentar chromatoids aggregated into sheaves. The majority of the cysts were eight-nucleated, but sometimes cysts with one or two nuclei and a great glycogen vacuole were present in abundance. In a few cases eight-nucleated cysts measuring from 9μ to 12μ were observed.

Besides the cysts of these two species we noted also cysts of *Endolimax nana, Iodamoeba bütschlii, Giardia intestinalis* and *Chilomastix mesnili. Balantidium* and *Coccidia* have never been found by us.

All the faeces were previously examined for helminthic infection.

RESULTS OF THE EXAMINATION

The results obtained from the examination of the three groups indicated above are set out in tabular form in Tables I to III.

TABLE I

Infection with intestinal parasites.

	Employees		Dispensary Patients			
			Adults		Children	
	Number	Per cent.	Number	Per cent.	Number	Per cent.
Total number examined	400	100	162	100	42	100
Infection with intestinal parasites in general	325	81·25	121	74·7	28	66·6
Protozoal infection	262	65·5	101	62·4	20	48·0
Helminthic infection	180	45·0	42	26·0	13	31·0
Protozoal infection without helminths	140	35·0	79	48·8	15	37·5
Helminthic infection without protozoa	58	14·5	20	12·3	8	19·0
Mixed helminthic and protozoal infection	122	30·5	22	13·5	5	11·0

TABLE II

Various protozoal and helminthic infections.

	Entamoeba histolytica.		Entamoeba coli		Endolimax nana		Iodamoeba butschlii		Giardia intestinalis		Chilomastix mesnili	
	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%
Employees	91	22·75	154	39·0	50	12·5	70	17·5	59	14·75	24	6·0
Adult patients ...	41	25·3	63	39·0	16	9·9	35	21·6	15	9·2	5	3·1
Child patients ...	4	9·5	9	21·5	2	4·8	2	4·8	8	19·0	1	2·4

	E. (Oxyuris) vermicularis		Ascaris lumbricoid.		Trichuris trichiura		Dibothrio- ceph. latus.		Taenia		Hymenolepis nana	
	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%
Employees	13	3·25	25	6·25	150	37·5	9	2·25	6	1·5	5	1·5
Adult patients ...	4	2·4	28	17·3	9	5·5	1	0·6	2	1·2	2	1·2
Child patients ...	2	4·8	11	26·2	...		...		...		...	

TABLE III

Pure and mixed protozoal infections.

	With one species		With two species		With three species		With four species		With five species	
	Total	%	Total	%	Total	%	Total	%	Total	%
Employees	136	52	83	31·0	29	11·0	11	4·1	5	1·9
Adult patients	47	46·5	36	35·6	14	13·8	3	2·9	1	1·0
Child patients	15	75·0	3	15	2	10	...		...	

An analysis of the protozoal infections of the most infected group (400 employees) is given in Tables IV to X.

TABLE IV.

Infections with *Entamoeba histolytica*.

Pure infection		Mixed infection							
		With one species		With two species		With three species		With four species	
Total	%	Total	%	Total	%	Total	%	Total	%
20	22·1	37	40·5	20	22·0	11	12·0	3	3·5

TABLE V

Infections with one species.

Entamoeba histolytica	Entamoeba coli	Endolimax nana	Iodamoeba butschlii	Giardia intestinalis	Chilomastix mesnili
20	60	13	16	23	4

TABLE VI

Infections with two species.

E. b. E. c.	E. c. I. b.	E. c. G. i.	E. b. E. n.	E. b. I. b.	E. c. E. n.	E. c. Ch.m.	E. n. I. b.	E. n. G. i.	E. b. G. i.	E. b. Cb. m.	I. b. Cb. m.	G. i. Cb. m.
17	15	11	8	7	5	5	4	3	3	2	2	1

TABLE VII

Infections with three species.

E. b. E. c. I. b.	E. b. E. c. E. n.	E. b. E. c. G. i.	E. c. G. i. Cb. m.	E. c. I. b. Cb. m.	E. c. E. n. G. i.	E. b. E. c. Cb. m.	E. b. E. n. I. b.	E. b. E. n. Cb. m.	E. b. I. b. G. i.	E. b. I. b. Cb. m.
6	6	3	3	2	2	1	1	1	1	1

TABLE VIII

Infections with four species.

E. b., E. c., E. n., I. b.	E. b., E. c., E. n., G. i.	E. b., E. c., I. b., Cb. m.	E. b., E. c., I. b., G. i.	E. b., E. c., G. i., Cb. m.
4	2	2	2	1

TABLE IX

Infection with five species.

E. b., E. c., E. n., I. b., Cb. m.	E. b., E. c., I. b., G. i., Cb. m.
3	2

TABLE X

Showing findings in the two most infected cases.

1		Ox. v.	Tr. tr.	Dibot. latus.	E. b.	E. c.	E. n.	I. b.
2	Ox. v.	As. lumbr.	Tr. tr.	Hymen. nana	E. b.	E. c.	E. n.	G. i.

As has been mentioned above, all the individuals examined were questioned on the present state of their gastro-intestinal system. Amongst the 400 apparently healthy employees, 189 persons or 47·25 per cent. suffered from various intestinal disorders, such as diarrhoea, constipation, pains in abdomen, often in its right half, flatulence and distension of the abdomen and so on ; 43 individuals were found to be carriers of *E. histolytica*. The number of these carriers forms also 47·25 per cent. of the total number of individuals infected with this parasite.

Table XI shows the distribution of individuals infected with *E. histolytica* amongst the employees of various food-shops.

TABLE XI

	Total number examined	Number infected with *E. histolytica*	Per cent.
Administration	71	17	23
Employees in provision shops	89	16	18
Employees in butchers' shops	70	18	26
Employees in green-grocers' shops	75	16	21
Employees in fish shops	10	2	20
Employees in confectionery shops	20	6	30
Employees in bakers' shops	65	16	24
Total number	400	91	22·75

The results of the measurement of *E. histolytica* cysts are shown in the following table.

TABLE XII

Sizes of cysts	Number of cases		
	Employees	Adult patients	Child patients
6–8µ	18	14	3
6–10µ	17	6	...
6–12µ	9	2	...
6–14µ	2	...	...
8–10µ	18	4	...
10–12µ	6	3	...
8–14µ	4	...	...
8–16µ	1	...	...
10–12µ	13	12	1
10–14µ	3	...	...

This table shows that amongst the employees cysts of 6μ to 10μ were found in 53 cases or in 60 per cent., and large cysts of 12μ, 14μ and 16μ in 38 cases or in 40 per cent. If we divide all the cases into two groups—one with cysts measuring less than 10μ, and the other with cysts from 10μ to 16μ, the first group will give 20 per cent. (18 cases) and the second 80 per cent. (73 cases) of the total number (91 cases) of employees infected with this parasite. The cases showing cysts of similar sizes were found as often as cases with cysts of different sizes. Thus there were 18 cases with cysts measuring from 6μ to 8μ, 18 cases with cysts from 8μ to 10μ, and 13 cases with cysts from 10μ to 12μ, which gives the total number of 49 cases or about 55 per cent. The remaining 42 cases or about 46 per cent., had cysts of different sizes. An extreme variation in size (6μ to 14μ or 8μ to 16μ) was observed in 22 cases or about 24 per cent. In the group of adult dispensary patients, infections with *E. histolytica* cysts of similar size were found in about 75 per cent. of the cases.

DISCUSSION OF RESULTS

The general infection with intestinal parasites amongst the examined groups of population reaches a very high percentage (66·6 for children, 74·7 for adult dispensary patients, and 81·25 for employees). So far as these figures were obtained from a single examination, the real figures must be still higher and it probably may be said, without exaggeration, that 100 per cent. of these groups of population are infected with intestinal parasites. As elsewhere the percentage of the protozoal infection is higher than that of the helminthic infection.

Of these three groups the first two (employees and adult dispensary patients) give very similar figures both for general infection and infections with different species. The cause seems to lie in the fact that the two groups consist of individuals with similar conditions of health. As has been mentioned above, almost 50 per cent. of the employees complained of gastro-intestinal disorders. Bearing in mind the insufficiency of the anamnestic data we may say that the majority of these apparently healthy individuals actually suffered from the same diseases, which affected the adult dispensary patients of our group.

Some figures seem to indicate that in these chronic disorders of the intestinal tract the protozoa play a larger rôle than the helminths. The percentage of protozoal infections without helminths is higher (48 and 35 per cent.) and the percentage of helminthic infections without protozoa is a little lower (12 and 14 per cent.) amongst the adult dispensary patients than amongst the employees.

The children group gives a somewhat different picture. Although the protozoal infection is also higher than the helminthic, the figures for the latter are higher than for the group of adults (19 and 12, 31 and 36), while those for the protozoal infection are lower (48 to 62·5, 37·5 to 48·8). A still greater difference is to be found in the figures concerning pure and mixed infections. Whilst in the adult group we find chiefly mixed infections (53·5 per cent.), amongst the children pure infections constitute 75 per cent. of cases.

As a rule the percentage of infection with different species for children is also lower than for adults; the infection with *Giardia intestinalis* forms the only exception, giving for children a much higher

percentage than for adults (19 and 9 per cent.). This fact seems to exist everywhere and confirms the opinion that this parasite is much more pathogenic for children than for adults.

The results obtained from our investigation greatly differ from the results of the surveys conducted in England, the United States of America, and other countries, as they show a much higher percentage of infection with *Iodamoeba bütschlii* (17·6 per cent.). This parasite in our country seems to be even more widespread than such a common parasite as *Endolimax nana* (12·5 per cent.). Another point should be noted. With regard to the number of cysts the infection with *Iodamoeba bütschlii* is considerably greater than all the other protozoal infections (except perhaps single cases of *Giardia intestinalis*). These cysts were sometimes present in extremely large numbers (15 to 20 and more cysts in each microscopic field).

It is obvious that the infection with *Entamoeba histolytica* constitutes the most important part of the general protozoal infection. Our figures resulting from a thorough and careful but single examination, have to be doubled at least in order to obtain a result which shall better approximate precision.

This doubling would give an exceedingly high percentage of *Entamoeba histolytica* infection amongst apparently healthy individuals. As we know that the percentage of *E. histolytica* infection may vary greatly for different groups of the population, we think it necessary to emphasize the urgency of further investigations in this direction in order to solve the question, whether the high percentage of this infection happens to be a characteristic feature of the examined groups of population only or whether it is common to all the inhabitants of Leningrad. The future work of the Department of Parasitology must give an answer to this important question.

No contradiction will be found between the prevalence of the dysenteric amoebae and the circumstance that only 47·25 per cent. of *E. histolytica* carriers complained of gastro-intestinal disorders, if we bear in mind two facts : (1) the insufficiency of anamnestic data, and (2) the existence of the so-called healthy carriers of *E. histolytica* who have no symptoms of gastro-intestinal trouble at all.

Table VII indicates that there is no connexion between any particular food trade and infection with *E. histolytica*. On the

other hand, whilst the average percentage of this infection among all the employees of food-shops amounts to 22·75 per cent., different food-shops show a much higher percentage of infected employees. For instance, the food-shop No. 1 has 66·6 per cent. of *E. histolytica* carriers. The difference is still greater for some other protozoa. Thus *Giardia intestinalis*, with an average percentage of infection 14·75 in the food-shop No. 20, gives 64·3 per cent.

The absence of connexion between *E. histolytica* infection and any particular food-trade and the obvious existence of some reservoir of this infection seem to indicate that *E. histolytica* infection, as is probably the case in protozoal infections in general, is propagated by means of contact and that direct, inter-human contagion is more important than the indirect infection by food or drink.

It is interesting to note that all the adult dispensary cases of *E. histolytica* had quite different clinical diagnoses, among which we can find even such as pregnancy, lumbago, spasm of thick gut and neurasthenia, and that none of these cases had been diagnosed as amoebiasis. In the majority of cases there was a diagnosis of helminthic infection, although only in eight of these eighteen cases helminths were found (*Oxyuris*, *Ascaris* and *Trichuris*).

Among child patients, cysts of *E. histolytica* were discovered in four cases, namely in one case of acute icterus (aged 7 years), in one case of anaemia (aged 5 years) and in two cases of acute blood-diarrhoea (one aged 9 months and the second aged 16 months).

In all the 45 dispensary cases of *E. histolytica* infection there were previous histories of chronic intestinal disorder.

The evidence resulting from the measurement of *E. histolytica* cysts rather contradicts Brumpt's suggestion of the existence of non-pathogenic *Entamoeba* with small four-nucleated cysts. On the other hand, the presence in half of the cases of cysts of different sizes seems to confirm the view of Dobell, Boeck and others, on the existence of different races of one *E. histolytica*.

SUMMARY

1. Six hundred and four individuals were examined in Leningrad for intestinal parasites. Each case had only one examination.

2. Amongst the group of 400 food-employees, 262 (65·5 per cent.)

were found to be infected with various protozoa. *E. histolytica* was discovered in ninety-one cases (22·75 per cent.).

3. One hundred and eighty-nine (47·25 per cent.) of these employees suffered from various gastro-intestinal disorders.

4. Of 162 adult non-dysenteric patients treated in the dispensary for various gastro-intestinal disorders, 101 (62·4 per cent.) were found to be infected with various protozoa. *E. histolytica* was discovered in forty-one cases (25·3 per cent.).

5. An investigation of forty-two child dispensary patients suffering from various gastro-intestinal disorders showed that twenty cases (48 per cent.) were infected with various protozoa. In four cases (9·5 per cent.) *E. histolytica* infections were found.

ENTAMOEBA HISTOLYTICA AND OTHER PROTOZOA IN TWO HUNDRED AND TWENTY-FIVE CASES OF ACUTE AESTIVAL DIARRHOEA ADMITTED TO THE BOTKIN BARRACKS HOSPITAL AT LENINGRAD (U.S.S.R.)

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The problem of aestival blood diarrhoea is still unsolved: notwithstanding the attention given to it during a long period of time by large circles of physicians, the true nature of this disease remains unknown. Many attempts to find its specific bacterial agent have given no positive results. Failure to find the cause of it seems to lie in the exclusive attention drawn to the bacteriological side of the question ; under the general grouping of aestival blood diarrhoea diseases with different etiologies are undoubtedly included.

The widespread presence of *Entamoeba histolytica* and other intestinal protozoa amongst the apparently healthy population of temperate countries suggests that these parasites must play a great rôle in the occurrence of intestinal diseases. But until the present time this question has received little attention in the Russian médical literature. The few papers on this subject—almost all of them concerning the south regions of U.S.S.R.—often give quite contradictory results. This fact seems to be due in a considerable degree to the incorrect methods of investigation employed.

The absence of such investigation conducted with correct methods for the temperate regions of U.S.S.R., and the widespread increase of *E. histolytica* and other intestinal protozoa among the population of these regions, rendered urgent the study of the rôle played by these parasites in the etiology of aestival diarrhoea, which in Leningrad sometimes takes the character of a real epidemic.

The investigation of this question constitutes one of the principal problems to be studied by the Department of Parasitology. According to this plan such investigation would have to consist in a careful clinical observation of a group of patients suffering from aestival diarrhoea with constant microscopical control of their faeces during all the course of the disease. As there were no technical facilities for the Department to conduct the work on such a scale, the investigation was limited to a single examination of as great a number as possible of such patients in a hospital, in order to study the intestinal protozoa, with a view to the discovery of cases of amoebic dysentery among those of aestival diarrhoea in Leningrad.

The work was conducted in the hospital of the Botkin Barracks, by kind permission of Professor G. A. Iwaschinzeff, M.D., the Chief of this hospital, to whom we express our indebtedness. We are also greatly indebted to M. A. Rapoport, M.D., Chief of the bacteriological laboratory of this hospital, who kindly gave us permission to conduct all the technical part of our work in this laboratory and supplied us with the information concerning the results of bacteriological examinations of all our cases. Finally we wish to acknowledge the help we received from the collaborator of this laboratory, Miss R. M. Pallerstein, D.Sc., in the collection of material and the careful preparation of many hundreds of films.

MATERIAL AND METHOD

During the period from July 1st to August 31st, 1929, 225 patients of the Botkin hospital for acute intestinal disorders were examined. In a great majority of cases these individuals were workers and employees who had resided at Leningrad for many years. These patients were admitted to the hospital with various diagnoses (see Table II below). The faeces in all the cases were also examined bacteriologically.

The material for our examination was collected during different periods of the disease and only in a few cases did we obtain it at the beginning of the disease, before the treatment had commenced. This circumstance had certainly a specific influence on the results of the investigation, but technical causes did not allow us to elude it.

From the beginning of our work we found that the usual procedure of sending the specimens from the ward to the laboratory was quite unsatisfactory. As a rule the material was brought too late, some hours after dejection, when the motile stages of the protozoa had already considerably degenerated and were often quite unfit for diagnosis, especially for the most important diagnosis of *E. histolytica* and *E. coli*.

Therefore, a short time after we began our work, another arrangement was made to secure the collection of quite reliable and fresh stools. The person in charge of the collection of specimens personally visited the barracks, and prepared films from faeces immediately after they had been passed. They were at once fixed by Schaudinn's fixative with 5 per cent. of acetic acid and stained by Heidenhain's iron haemotoxylin. In the majority of cases the stools were examined only once for each patient, but about 20 per cent. were examined from two to six times. In each case two films, measuring 18 mm. by 36 mm. and 18 mm. by 54 mm. were prepared. All the films were examined several times (an average of not less than 10 to 15 minutes being devoted to each film) with fluorite immersion Zeiss 100 objective and compensating eye-piece 7 ×.

The cases, in which we found (1) *Entamoeba* with red blood corpuscles, (2) cysts of *E. histolytica* (associated or not associated with motile stages) and (3) motile stages of *Entamoeba* without red blood corpuscles and cysts, but distinguishable themselves by a complete absence of any ingested material, by a sharp differentiation of plasma into endo- and ecto-plasma and by a typical structure of the nucleus,* were noted as *Entamoeba histolytica*. All the doubtful cases, which did not show clearly the features mentioned above, were noted as *E. coli*.

In a few cases *Enteromonas hominis* and *Embadomonas intestinalis* seemed to be discovered. But they were not noted, as the films were not correctly differentiated and therefore these parasites could not be reliably diagnosed.

All the *E. histolytica* and *E. coli* found were measured with a micrometric eye-piece.

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* A delicate nuclear membrane with single layer of minute chromatin grains on its inner surface, a minute karyosoma situated in the centre of the nucleus and no chromatin between the karyosoma and the nuclear membrane.

RESULTS OF EXAMINATION

Out of 225 patients, 110 (or 49 per cent.) were found to be infected with some of the protozoal organisms. In Table I an analysis of positive cases is given and the incidence of protozoal infection shown.

TABLE I

Showing incidence of protozoal infection found.

	Ent-amoeba histolytica	Ent-amoeba coli	Endo-limax nana	Iod-amoeba bütschlii	Dient-amoeba fragilis	Giardia intestinalis	Chilo-mastix mesnili	Tricho-monas hominis.	Balan-tidium coli
Number of cases infected ...	32	54	26	27	1	5	17	2	1
Percentage of total cases examined ...	14·2	24·0	11·5	12·0	0·4	2·2	7·5	0·9	0·4
Pure infection	14	25	11	10	...	4	7	1	1
Mixed infection ...	18	29	15	17	1	1	10	1	...

One patient frequently harboured two or more protozoa. Some idea of the commoner mixed infections found may be gained from the list given at the end of this report.

E. histolytica was found with red-blood corpuscles in 13 cases, in 8 cases associated with cysts, and in 11 cases without cysts and red blood corpuscles.

The bacteriological examination of faeces showed positive results for bacillary dysentery in 22 cases (9·8 per cent.). But *E. histolytica* was also found in only one of these cases (Case 37).

TABLE II

Diagnosis	Total number of cases	Bacillary dysentery		Amoebic dysentery	
		Total number	Per cent. of total cases	Total number	Per cent. of total cases
Dysentery	8	3	37·5	1	12·5
Colit. and enterocol. haemorrh. ...	111	8	7·2	20	18·0
Colitis ac.	41	6	14·6	3	7·3
Entero-colitis ac.	20	3	15·0	5	25·0
Gastro-entero-colitis	6	...	...	...	...
Gastro-enteritis ac.	24	...	...	2	8·3
Enteritis ac.	11	2	18·2	1	9·0
Gastritis ac.	1	...	...	...	...
Typhus abdominalis	3	...	...	...	...
Total	225	22	9·8	32	14·2

In a great majority of cases infected with *E. histolytica*, the patients were workers who had resided at Leningrad for many years ; three individuals had arrived from Pskoff and Witebsk districts. Two patients had lived recently in the Caucasus and in Turkestan.

With regard to the course of *E. histolytica* infection it should be noted that in a large number of the cases, the disease was of a short duration, as shown below.

Left hospital 7 days or less   ...   . 8 persons.

,,   ,,   8 to 14 days   14   ,,

,,   ,,   15 to 21 days   6   ,,

,,   ,,   22 to 30 days   2   ,,

,,   ,,   31, 42, 52 days   3   ,,

It must be noted that in some cases the patients leaving the hospital still had some symptoms of the disease, such as a soft unformed stool, pain or tenderness in the region of the thick gut, pains in abdomen and sometimes even continuous diarrhoea.

In the anamnesis of many cases frequent intestinal disorders have been noted.

In six fatal cases examined only once each, no *E. histolytica* was found. In one we found *Balantidium coli*, which was probably the cause of death. In the remaining five cases, although the bacteriological and protozoological examination gave negative results, the post-mortem findings do not exclude the possibility of amoebic dysentery. The epicrisis in these cases was : (1) dysenteria, (2) colitis ulcerativa, and (3) entero-colitis necrotica diphtheritica.

DISCUSSION OF RESULTS

The evidence obtained from our examination leaves no room for any doubt concerning the existence of amoebic dysentery amongst the aestival blood diarrhoea in Leningrad. There is also no doubt that the percentage of such cases must be considerable. The percentage shown by our investigation (14·2) is much lower than the real one. The examination of faeces a short time after enemas of oil or after some remedy administered per os could not fail to reduce the percentage of positive findings ; still greater importance had the fact of a single examination. About 20 per cent. of the cases were examined two, three, four and five times each ; consequently, the percentage of positive findings in this group of patients was considerably increased. Thus : in 173 cases examined once each, *E. histolytica* was found in 12·1 per cent. ; in 41 cases examined twice each, *E. histolytica* was found in 19·5 per cent. ; in 11 cases examined from three to five times, *E. histolytica* was found in 27·7 per cent.

Bearing in mind that, according to Wenyon, Boeck and others, a single examination discovers only about one-third of the total number of real infections, it may be acknowledged that in Leningrad about half of all the individuals suffering from acute aestival diarrhoea are infected with *E. histolytica*. The widespread prevalence of *E. histolytica* amongst the dispensary patients and employees of co-operative food-shops in Leningrad, discovered by a special investigation, confirms the correctness of this conclusion.

It is possible that some cases, in which *E. histolytica* was found, are not cases of true dysentery. Cases in which *E. histolytica* did not contain red blood corpuscles could be only carriers of

E. histolytica having no active amoebic dysenteric process in progress and suffering from bacillary or other form of dysentery. But the 13 cases (5·8 per cent.) in which *E. histolytica* contained red blood corpuscles must be noted as cases of true amoebic dysentery. And therefore it should be stated that *not less than* 15 *to* 20 *per cent. of cases of acute aestival diarrhoea in Leningrad are cases of amoebic dysentery.*

The short duration of the disease sometimes observed in these cases does not contradict at all the fact of their amoebic etiology. A chronic course of disease with short timed relapses is characteristic of amoebic dysentery. Such was the first case of this disease described by Lösch (blood diarrhoea during a fortnight, after which the patient left hospital in satisfactory condition ; the last and fatal relapse happened after some months.)

It may appear strange that whilst amoebic dysentery gives such a high rate of occurrence, liver abscess is said to be absent. It is nevertheless dangerous to speak of complete absence, because the statistical data on the occurrence of amoebic liver abscess collected by Voliansky in 1898, show that it was found in about 0·3 per cent. of autopsies examined in the hospitals of St. Petersburg. According to the verbal communications of some Leningrad pathologists, liver abscesses are observed at the present time in rare cases and almost always in combination with ulcerative processes in the appendix and caecum. The nature of these abscesses remains unknown or, rather, not investigated. We are convinced that a suitable examination would find amongst these cases a considerable percentage of true amoebic abscesses.

The comparative rareness of amoebic liver abscesses in temperate countries does not disprove the fact that amoebic dysentery is widespread. The conception that amoebic liver abscess is a common complication of amoebic dysentery arose as the result of observations on European patients in the tropics. But according to Cort (1928), in Siam, and to Williams, Wildman and Curtis (1929), in Haiti, liver abscess occurs very rarely amongst natives, although amoebic dysentery is much more widespread among natives than among Europeans (according to Cort, amoebic liver abscesses among European and native soldiers were found in the proportion of 95·2 : 4·8). *A priori* we cannot exclude the possibility that the

cause of the scarceness of these abscesses in temperate countries lies in the fact that the population consists largely of native elements.

Finally, as regards autochthonous cases of amoebic dysentery, it must be noted that there were undoubtedly such cases in our material. The possibility of these cases in temperate countries in general has been established by Wenyon, Dobell and others. As to Leningrad, the first case of amoebic dysentery described in the literature, the so-called case of Lösch, was a true autochthon case. But it is very difficult at the present time to establish the fact that amoebic dysentery is autochthonous on account of a very much developed migration of population. The moving of great numbers of people from north to south and vice versa (business voyages, travels, visits to southern health resorts and so on), neutralise in this respect the differences between the north and the south population. It is quite probable that the increase of E. histolytica infection observed at present in the temperate countries may find an explanation in these facts.

We shall not try to analyse in detail the relation of other intestinal protozoa to acute intestinal diseases, but we think it necessary to note one fatal case of balantidiosis. The anamnesis of this patient showed a previous contact with pigs. Repeated bacteriological examinations of her faeces gave negative results. A protozoological examination made on the day of death discovered a great number of *Balantidium coli*. The post mortem diagnosis was of a diphtheto-necrotic colitis. This case is very important because it clearly demonstrates *the absolute necessity of not only bacteriological but also protozoological examination in all the cases of acute intestinal disorders.*

SUMMARY AND CONCLUSIONS

1. From July 1st to August 31st, 225 patients lying in the Botkin Barracks Hospital, in Leningrad, with various acute intestinal diseases were examined, and 110 (49·0 per cent.) were found to be infected with various protozoa.

2. *E. histolytica* was found in 32 cases (14·2 per cent.) ; in 13 of these cases (5·8 per cent.), *E. histolytica* contained red blood corpuscles.

3. As the majority of cases (173) was examined only once each, the real figures of *E. histolytica* infection should be considerably increased ; it may be said that *about 15 to 20 per cent. of acute aestival diarrhoea cases are cases of true amoebic dysentery*.

4. Bacillary dysentery amongst these 225 cases was found in 22 cases (9·8 per cent.) and only in one case was it combined with amoebic dysentery.

5. The prevalence of the intestinal protozoa in general, and of *E. histolytica* especially, makes a protozoological examination of all acute intestinal disorders absolutely indispensable.

LIST OF CASES IN WHICH PROTOZOA WERE FOUND.

	Record number	Diagnosis	E. histolytica	E. coli	E. nana	I. butschl.	D. fragil.	G. intest.	Cb. mesnili	T. hominis	B. coli
1	1	Colitis haemor.	...	...	...	...	...	...	...	...	+
2	2	,,	..	+	...	...	...	+	...	...	...
3	13	,,	...	+	...	...	...	...	...	...	...
4	15	,,	..	+	...	+	...	...	...	...	...
5	18	,,	+	+	...	+	..	...	...	...	...
6	30	,,	...	+	...	...	...	...	...	...	...
7	31	,,	+	...	...	...	...	..	...	...	...
8	37	,,	+	+	+	...	...	...	+	...	...
9	41	,,	+	...	...	...	...	...	...	...	...
10	42	,,	..	...	...	+	...	...	...	...	...
11	44	,,	+	...	...	..	...	...	...	...	...
12	46	,,	+	+	+	...	...	...	+	...	...
13	47	,,	+	..	..	...	...	...	...	...	...
14	66	,,	+	...	...	...	...	...	...	...	...
15	77	,,	+	+	+	+	...	...	..	...	...
16	86	,,	+	+	...	...	...	...	+	...	...
17	88	,,	..	..	+	...	...	...	...	...	...
18	89	,,	..			...	...	...	...	...	...	...
19	101	,,	...	+	..	...	...	...	...	...	...
20	107	,,	...	...	+	...	...	...	...	...	...
21	112	,,	+	+	+	...	...	...	...	...	...
22	116	,,	...	+	...	+	...	...	...	...	...
23	139	,,	...	+	...	+	...	...	...	...	...
24	161	,,	+	...	...	...	...	...	...	...	...
25	163	,,	...	+	...	...	...	...	...	...	...
26	167	,,	...	...	...	+	...	...	+	...	...
27	178	,,	...	...	...	+	...	...	...	...	...
28	209	,,	+	...	..	...	...	...	...	...	...
29	210	,,	...	+	...	...	...	...	...	...	...
30	216	,,	...	...	+	...	...	...	...	...	...
31	235	,,	...	+	...	...	...	...	...	...	...
32	236	,,	...	...	...	+	...	...	...	...	...
33	252	,,	...	+	...	...	...	...	+	...	...
34	253	,,	...	...	+	+	...	...	+	...	...
35	258	,,	...	...	+	...	...	...	...	...	...

LIST OF CASES IN WHICH PROTOZOA WERE FOUND—*continued.*

	Record number	Diagnosis	*E. histol.*	*E. coli*	*E. nana*	*I. butschl.*	*D. fragil.*	*G. intest.*	*Cb. mesnili*	*T. hominis*	*B. coli*
36	264	Colitis haemor. ...	+	+	+	...	...	...	...	...	...
37	267	,,	...	+	...	...	...	...	...	...	...
38	268	,,	...	...	...	...	...	...	+	...	...
39	271	,,	+	...	+	...	...	...	...	...	...
40	296	,,	...	+	...	...	...	...	...	...	...
41	298	,,	...	+	...	...	...	...	...	...	...
42	312	,,	+	...	...	...	...	...	...	...	...
43	24	Entero-colitis haem. ...	+	+	...	...	...	...	+	...	...
44	54	,,	...	+	...	...	...	...	...	...	...
45	69	,,	...	+	...	...	...	...	+	...	...
46	186	,,	...	...	+	+	...	...	...	...	...
47	224	,,	...	...	+	...	...	...	...	...	...
48	225	,,	...	...	...	...	...	...	+	+	...
49	226	,,	...	...	...	...	...	+	...	...	...
50	230	,,	...	...	+	...	...	...	...	...	...
51	241	,,	...	...	...	+	...	...	...	...	...
52	278	,,	...	+	...	...	...	...	...	...	...
53	293	,,	+	+	...	...	...	...	...	...	...
54	299	,,	+	+	...	...	...	...	...	...	...
55	300	,,	+	...	...	+	...	...	...	...	...
56	304	,,	...	+	...	...	...	...	...	...	...
57	307	,,	+	...	...	...	...	...	...	...	...
58	96	Dysenteria	...	...	...	...	...	...	+	...	...
59	242	,,	...	+	...	...	...	...	...	...	...
60	303	,,	+	+	...	...	...	...	...	...	...
61	8	Colitis acuta	...	+	...	...	...	...	...	...	...
62	9	,,	...	...	...	...	...	...	+	...	...
63	14	,,	...	...	+	...	...	...	...	...	...
64	17	,,	...	...	...	...	...	...	+	...	...
65	21	,,	...	...	+	...	...	...	...	...	...
66	95	,,	...	+	...	+	...	...	...	...	...
67	106	,,	+	...	+	...	...	...	...	...	...
68	110	,,	...	+	...	+	...	...	...	...	...
69	114	,,	...	...	...	+	...	...	...	...	...
70	121	,,	+	+	+	+	...	...	...	...	...
71	154	,,	...	+	...	+	...	...	...	...	...
72	162	,,	...	+	...	...	...	...	...	...	...
73	169	,,	...	+	...	...	...	...	...	...	...
74	181	,,	...	...	...	...	...	...	...	+	...
75	247	,,	...	+	...	...	...	...	...	...	...
76	276	,,	...	...	...	+	...	...	...	...	...
77	288	,,	...	+	...	...	...	...	...	...	...
78	290	,,	...	...	...	+	...	...	...	...	...
79	305	,,	+	...	...	...	...	...	...	...	...
80	5	Entero-colit. ac. ...	+	+	...	...	...	...	...	...	...
81	53	,,	+	...	...	...	...	...	...	...	...
82	138	,,	...	...	+	...	...	...	...	...	...
83	150	,,	...	+	...	...	...	...	...	...	...
84	160	,,	...	...	...	...	...	+	...	...	...
85	232	,,	+	+	...	+	...	...	...	...	...
86	250	,,	+	...	...	...	...	...	...	...	...
87	259	,,	...	...	...	...	...	...	+	...	...
88	269	,,	+	...	...	+	...	...	...	...	...
89	270	,,	...	+	...	+	...	...	...	...	...
90	279	,,	...	+	...	+	...	...	...	...	...

LIST OF CASES IN WHICH PROTOZOA WERE FOUND—*continued*.

	Record Number	Diagnosis	E. histol.	E. coli	E. nana	I. bütschl.	D. fragil.	G. intest.	Cb. mesnili	T. hominis	B. coli
91	3	Gast.-ent. colitis	...	...	...	...	...	...	+	...	...
92	4	,,	...	...	...	...	...	...	+	...	...
93	199	,,	...	+	...	...	...	...	...	...	...
94	10	Gast.-enter. ac.	...	...	...	...	...	+	...	...	...
95	26	,,	...	...	+	...	...	...	...	...	...
96	32	,,	...	+	+	...	...	...	...	...	...
97	40	,,	...	+	+	...	...	...	...	...	...
98	85	,,	...	...	+	...	...	...	...	...	...
99	141	,,	...	+	...	+	...	...	+	...	...
100	143	,,	+	...	+	...	...	...	...	...	...
101	144	,,	...	...	...	...	...	+	...	...	...
102	314	,,	+	...	...	...	...	...	...	...	...
103	27	Enteritis acuta	...	+	...	...	...	...	...	...	...
104	62	,,	...	+	+	...	...	...	...	...	...
105	67	,,	...	+	+	...	...	...	...	...	...
106	75	,,	...	...	...	+	...	...	...	...	...
107	187	,,	+	...	...	...	...	...	...	...	...
108	243	,,	...	+	+	+	+	...	...	...	...
109	227	Gastritis acuta	...	+	...	...	...	...	...	...	...
110	254	Typhus abdom.	...	...	...	+	...	...	...	...	...

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ON THE IDENTIFICATION AND LIFE HISTORY OF *ECHINOSTOMUM RECURVATUM* VON LINSTOW, 1873

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PLATE III

In 1892, Sonsino described *Cercaria agilis* from *Physa alexandrina* fully, but he failed to observe that it had a crown of spines, and took its absence as a distinctive feature. He also saw them encysted in the tissues of different snails and made out the crown of spines in the encysted ones. Further, he fed rabbits and geese on snails containing the cysts, and got a few adult echinostomes after fifteen days. He described these adults as *Echinostomum recurvatum* von Linstow, 1873, and could distinguish 42 to 44 spines in the crown at the cephalic end.

In making a series of examinations in Bulinus of different species, I came across large numbers of these encysted cercariae in the tissues of the molluscs, particularly in the branchial tubes. Each cyst is spherical and transparent, and measures about 190μ in diameter. Apart from the genitalia, the larva when brought out of the cyst does not show any features more than those of the free cercaria; and a crown of 43 to 45 spines can be made out in the free cercaria and the encysted ones. These cysts were also met with in a large number of different snails free from rediae such as Vivipara, Cleopatra, Planorbis, Lenistes and Melania.

The free cercaria (*C. agilis*), together with the other larval stages, i.e., immature and mature rediae containing free and encysted cercaria were found in large numbers in *Bulinus contortus, B. dybowski* and *B. innesi*.

Repeating the feeding experiments on different animals such as dogs, white and wild rats, I found ova in the faeces after an interval of seven days, and collected large numbers of the adult trematodes after varying intervals from seven days to six months.

The trematodes from the different animals varied in size according to the length of the time they passed in their final hosts. They were of the same morphology and had the same number of spines as those described by Sonsino.

The original description of *E. recurvatum* by von Linstow, 1873, was not available, but the type species was kindly lent by the authorities of the Berlin Museum. These specimens were identical in morphology with the specimens I have obtained from experimental animals fed on infected snails and with the type species, *Echinostoma aegyptiaca*, described by Khalil and Abaza (1924). The latter was met with in a rat caught in the P.H. Laboratories of Cairo, where there had always been a fair number of snails brought for research purposes.

Since the validity of *E. aegyptiaca* as a new species depends on the presence of this echinostomum in a new host (the rat), which willingly feeds on snails and can acquire the infection as easily as other animals, and since there is a striking morphological identity between it and *E. recurvatum* von Linstow, 1873, it is suggested that the species described by Khalil and Abaza (1924) should not be recognised as a distinct one from *E. recurvatum*

I am indebted to Professor Khalil for laboratory and library facilities and for many valuable hints.

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PLATE III

EXPLANATION OF PLATE III

Fig. 1.   Shows free *Cercaria agilis* from *Bulinus dybowski*.

Fig. 2.   A redia from the liver of an infected Bulinus, showing immature free cercariae and an encysted one.

Fig. 3.   An adult *E. recurvatum* from an experimentally infected dog one month after the infection.

All the drawings were made with the Camera Lucida.
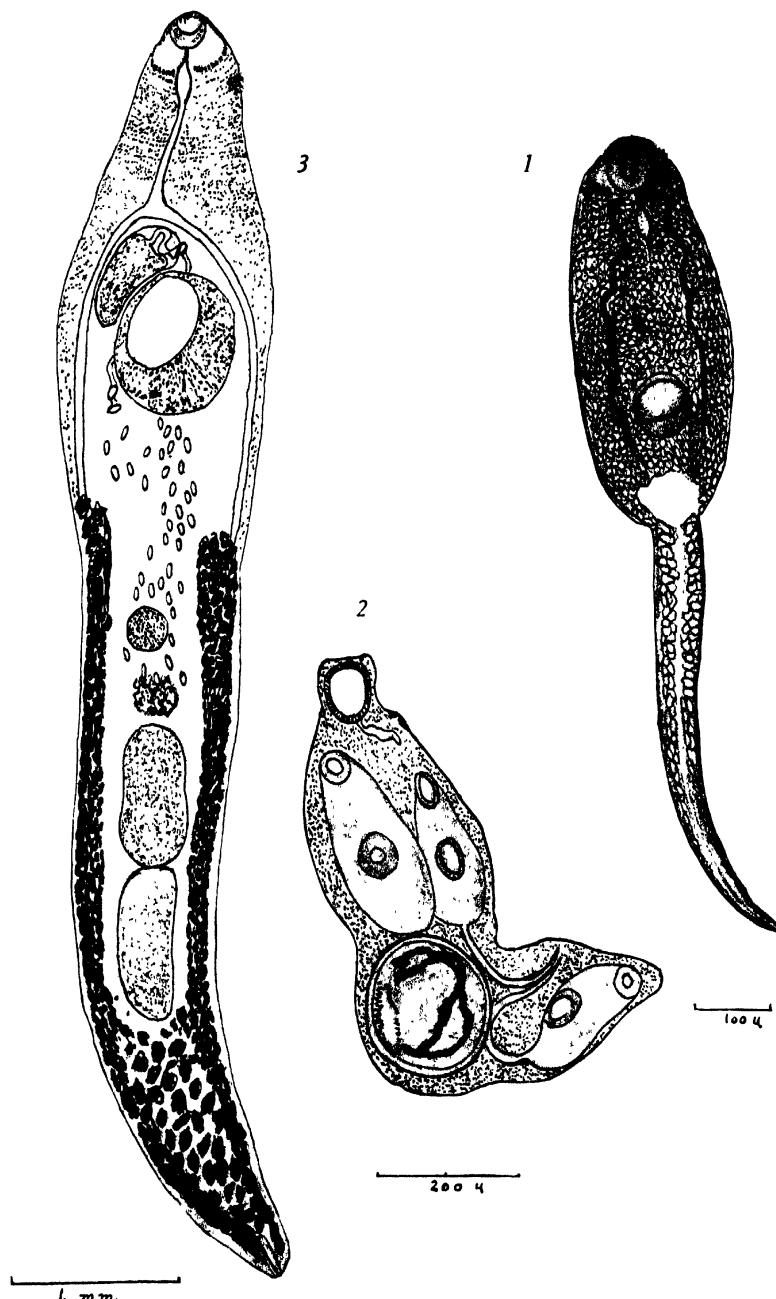
THE BEHAVIOUR OF INSECT
FLAGELLATES AND LEISHMANIAS
IN *PHLEBOTOMUS PAPATASII*

BY

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In previous papers (1927 and 1929) the behaviour of various strains of Leishmania in *P. papatasii* infected by feeding on emulsions of flagellates in inactivated rabbit blood was described.

L. tropica from cultures behaved similarly to *L. tropica* ingested from oriental sores. The flagellates multiplied, ascended the cardia and pharynx and in a few cases entered the proboscis.

Of four strains of *L. donovani* of Mediterranean origin, one remained in the stomach, three other strains and two strains of *L. tarentolae* behaved similarly to *L. tropica*. Two other strains of *L. donovani* from Naples were found to produce a low infection rate in *P. papatasii*, but if the infection was established, the parasites ascended to the top of the cardia and attached themselves to the rhabdorium.

Two strains of *L. donovani* from India attached themselves to the rhabdorium in the cardia if ingested in sufficient quantities, but the infection tended to die out.

A strain of *L. donovani* from China which was abnormal in that it attained a maximum size of only $11 \cdot 2\mu$ and was usually devoid of a flagellum or showed only a very short flagellum, remained in the stomach of *P. papatasii* even if ingested in doses of 8,000 parasites per feed.

One strain of *L. brasiliensis* which was abnormally small remained in the stomach, and another strain has been found to ascend and

attach itself to the rhabdorium near the oesophageal valve if ingested in sufficient quantities.

L. ceramodactyli was found to adopt a typical posterior position but the flagellates ascended the cardia, attached themselves to the rhabdorium and even entered the pharynx if the infection was very heavy.

The behaviour of *L. tropica* and *L. ceramodactyli* in *P. papatasii* was interpreted as showing a specific host-parasite relationship between the insect and the protozoon. The behaviour of the other normal strains is interpreted as proving that *P. papatasii* is not the usual vector but they still showed some specific characters in that they attached themselves to the rhabdorium of the cardia and entered the pharynx and buccal cavity. The specificity is quite definite in the case of *L. tropica,* for the behaviour of the flagellates is independent of the intensity of the infection produced in the sandfly. In other cases the specificity was of a lower order for it depended on quantitative factors such as the intensity of the infection produced. A strain of *L. agamae* David, tested in our laboratory by Dr. A. David, was found to be very irregular in its behaviour. In the majority of cases the flagellates confined themselves to the stomach, occasionally they descended into the hindgut and very rarely ascended the cardia and attached themselves to the rhabdorium.

There is a close biological relationship between strains which behave similarly in *P. papatasii.* Thus all the strains which tend to adopt an anterior position when established in the sandfly are closely related serologically ; *L. tropica, L. donovani, L. brasiliensis* and *L. tarentolae* contain group agglutinins.

Serologically they can all be readily distinguished from *L. ceramodactyli* and *L. agamae* and the two latter species have no group agglutinins. It is therefore of interest to note the behaviour of plant and purely insect flagellates which are biologically and serologically distinct from the Leishmanias. The following strains presented by Dr. J. G. Thomson were examined.

(1) *Herpetomonas culicidarum* (from *Culex pipiens*).

In four experiments, 28 sandflies, *P. papatasii* were fed through membranes on emulsions of *H. culicidarum* varying from 240 to 2,500 per 0·1 c.mm.

The sandflies were dissected from 2 to 13 days after the feed. All were positive and in every one the infection was confined to the stomach.

Six sandflies were re-fed on a human being but the re-feed did not influence the course of the infection. *In vitro* fresh human blood rapidly destroys *H. culicidarum* as well as *L. tropica*, but inactivated blood has no effect. As pointed out in a previous paper, re-feeding has no effect on the course of *L. tropica* infections in *P. papatasii*. The protection afforded by the midgut of *P. papatasii* against the lytic action of fresh blood is therefore not specific ; it depends on the rapid destruction of complement in the midgut and is the same for *H. culicidarum* as for *L. tropica*.

(2) *Herpetomonas oncopelti* (from *Oncopeltus fasciatus*).

In five experiments, a total of 35 sandflies, *P. papatasii* fed on emulsions of 600 to 1,200 flagellates per 0·1 c.mm., and were dissected from 2 to 13 days after the feed. All were positive. Re-feeding on a human being had no influence on the infection. In 18 sandflies, in addition to the flagellates in the stomach, round a-flagellar forms were seen in the stomach and hindgut. In the hindgut many of these forms were attached to the chitinous intima. The round forms tended to die out for they were not seen in the later infections. There is apparently little multiplication after 4 or 5 days, and the later infections were all slight.

(3) *Herpetomonas oncopelti* (from *Asclepias syriaca*).

In nine experiments, a total of 38 sandflies, fed on emulsions of 50 to 1,600 flagellates per 0·1 c.mm., and were dissected 2 to 12 days after the feed. Of these, 32 were positive. In 12 sandflies there were round forms in the stomach and hindgut as in the previous strain. Re-feeding had no effect on the infection. The infection became progressively smaller. This flagellate only produces slight infections in *P. papatasii*.

(4) *Herpetomonas lygaeorum*.

In four experiments, 27 sandflies fed on emulsions of 300 to 600 flagellates per 0·1 c.mm., and were dissected 1 to 12 days after the feed. Of these, 16 were positive. After 2 or 3 days a-flagellar forms were found in the hindgut. This flagellate multiplies very little in *P. papatasii* and produces slight infections.

(5) *Herpetomonas muscidarum.*

In seven experiments, 86 sandflies fed on emulsions of 250 to 1,400 flagellates per 0·1 c.mm., and were dissected 2 to 14 days later. Of these, 59 were positive. *P. papatasii* is an unsuitable medium for this flagellate. The infection diminishes continuously after the first few days and no positives were found after 7 days. The flagellates occasionally descend into the hindgut where they die.

In the above five strains there was no tendency to ascend the cardia. *H. culicidarum* behaved as in a culture tube, *H. muscidarum* gradually disappeared. In the case of the three other species there were no heavy infections such as those produced by *L. tropica*, which often distends the whole upper part of the midgut by a solid mass of flagellates. Round forms appeared in the hindgut but they were apparently in an unsuitable medium and died out.

The development of the four insect flagellates and the plant flagellate in an artificial host, *P. papatasii*, which is biologically very distant from their true host, may be taken as an example of non-specific behaviour.

The behaviour of *L. donovani* and *L. tropica* in the bed bug is quite different from their respective development in *P. argentipes* and *P. papatasii*, and also forms a typical example of non-specific behaviour. It can be best compared to the case of *H. muscidarum* and *P. papatasii*, where there is certainly no natural host-parasite relationship.

The above observations support the view that the multiplication and behaviour of *L. tropica* in *P. papatasii* is no mere accident, but points to a host-parasite relationship between the two.

We have to thank Dr. J. G. Thomson, of the London School of Tropical Medicine, for the gift of strains.

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THE INOCULATION OF CANINE CUTANEOUS LEISHMANIASIS INTO MAN AND THE BEHAVIOUR OF VARIOUS STRAINS OF LEISHMANIA IN MICE

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PLATES IV, V

In a previous paper (1929) one of us (A.) discussed the distribution of Leishmaniasis in man and dog. It was pointed out that centres of Leishmaniasis, both cutaneous and visceral, were of two types, one in which human beings and dogs were infected, and the other in which human beings only were infected, dogs rarely or never contracting the disease. In the former type of centre, infants under one were frequently infected. This curious distribution of Leishmaniasis requires investigation. Several explanations were put forward and discussed, e.g., in centres of the second type the insect vector may not bite dogs at all, or successful transmission of the parasite by the insect depends on a factor such as crushing, which is absent in dogs or children under twelve months. In centres of the first type it is obvious that transmission must be by bite, for dogs and babies cannot crush sandflies.

Before any hypothesis put forward to explain the distribution of Leishmaniasis in man and dog can be considered, the relationship between human and canine Leishmaniasis must be investigated experimentally. Up to the present this has been attempted by inoculations of human Leishmaniasis into dogs. It was found that strains of human Leishmania even from centres where the disease is confined to man, produce a disease in dogs very similar to the

one occurring naturally in these animals. This evidence, though suggestive, is incomplete and the matter must be settled definitely by experiments with canine *Leishmania* on man. Working with a strain of canine cutaneous Leishmaniasis from Baghdad, we were able to produce cutaneous Leishmaniasis in a human being.

HISTORY OF STRAIN

The strain was isolated by Mr. McHattie, in September, 1928, in Baghdad, from a naturally occurring cutaneous lesion in a dog. The strain arrived in Jerusalem in June, 1929, and was maintained since on Locke-Serum-Agar.

EXPERIMENT I.

22.7.29. Culture material was inoculated into two points on the left arm of a volunteer.

22.7.29. Flagellates from three laboratory bred sandflies, Nos. 7106, 7108 and 7112, infected with the same strain by feeding through a membrane, were inoculated into two points on the right arm of the same volunteer. (The whole of the midgut of all three sandflies was inoculated.)

These three sandflies were chosen from the following experiments :

EXPERIMENT 454.

An emulsion in inactivated rabbit blood of culture (ten days old), 8,000 flagellates per c.mm., i.e., 800 flagellates per feed of *Phlebotomus papatasii* was used.

14.7.29. Fourteen females of *P. papatasii*, laboratory bred, fed on this emulsion through a membrane of rabbit skin.

Result.

One sandfly, No. 7099, dissected after five days. Cardia and stomach infected.

Two sandflies, Nos. 7100 and 7101, dissected after seven days. One sandfly positive in cardia and stomach.

Eleven sandflies, Nos. 7102 to 7113, dissected after eight days. Six sandflies positive in cardia and stomach.

In most of the sandflies the infection was very heavy, the cardia being choked with flagellates.

RESULTS OF THE INOCULATION EXPERIMENT

There was some irritation at the points inoculated with flagellates from the sandflies, and two small scaly papules developed. On 27.8.29 the papules were examined and no L.D. bodies were found. They disappeared completely by September, 1929.

On the site of the inoculations with culture on the left arm, two papules were observed at the beginning of September, 1929.

13.9.29. Scrapings of the papules showed numerous L.D. bodies. Cultures on Locke-Serum-Agar were positive. The papules grew and by 12.2.30 were about 8 mm. in diameter and irregular in outline (Pl. IV, fig. 2.)

12.2.30. One papule was excised for histological examination.

EXPERIMENT 2.

22.7.29. The midguts of two sandflies (Nos. 7102 and 7104, from Experiment 454), with a heavy midgut infection, were inoculated into two points on the left arm of a volunteer spontaneously cured of an experimental infection with *L. tropica*. This experimental infection has been described (1925 and 1926). The experiment with sandflies Nos. 7102 and 7104 was performed about eighteen months after spontaneous cure.

Result. There was a local reaction on both points of the inoculation. Two scaly papules appeared after a few days and remained for two weeks. No L.D. bodies were found. Inoculation of living or dead culture of *L. tropica* always produces this reaction in the same volunteer.

The result of Experiment 1 was contrary to our expectations for a number of attempts (previously recorded) to infect human beings with cultures of *L. tropica* (Palestinian strains) all gave negative results, although we had positive results with cultures of Palestinian strains which had passed through sandflies (one case, 1927). We also had positive results with inoculations of flagellates from naturally infected sandflies (three cases, 1925 and 1926) and with inoculations of flagellates from laboratory bred sandflies which had fed on oriental sores (six cases from *P. papatasii*, 1927, and one case from *P. sergenti*, 1929).

We had, therefore, expected Experiment 1 to give either a

negative result on all sites of the inoculation or a positive result on the site of the inoculation with the infected sandflies.

We must bear in mind, however, that the strain used originated from Baghdad, and probably was transmitted by *P. sergenti*, whereas our previous conclusions had been based on a study of Palestinian strains whose natural vector is *P. papatasii*. As will be shown later, the strain used in Experiment 1 differed in some respects from Palestinian strains, but we do not consider these differences to be of specific value.

The result of Experiment 1 proves conclusively that there is a cutaneous Leishmaniasis common to man and dog. *Leishmania tropica* is, therefore, a naturally occurring parasite of dogs in Baghdad.

The relationship between human and canine visceral Leishmaniasis still remains to be investigated.

Cutaneous Leishmaniasis has not yet been found in dogs in Palestine, but this is not because the Palestinian strains are non-infective for dogs. We succeeded in infecting a dog on both ears by cultures of a Palestinian strain of *L. tropica* and by flagellates of the same strain passed through sandflies (*P. papatasii*).

The strain originated from a naturally infected sandfly (*P. papatasii* from Jericho), passed through (1) a human being in whom it produced a subcutaneous nodule, (2) another human being in whom it produced a papule, (3) cultures, (4) a mouse, and (5) cultures.

In order to produce a lesion in a dog with cultures it is necessary to employ large quantities ; in the successful experiment we employed three tubes of culture on Locke-Serum-Agar. Employing loop-fuls of culture we received negative results in two dogs.

HISTOPATHOLOGY OF THE LESION PRODUCED IN EXPERIMENT 1

The histopathology- is in general outlines similar to that of naturally occurring oriental sore in man. The lesion consisted of an infiltrated area in the Corium. Above the lesion the epidermis was atrophied, the rete mucosum being almost entirely destroyed. Between a part of the lesion and the atrophied skin there was a space (Pl. IV, fig. 3, *b*) filled with blood-stained fluid containing a large

number of free L.D. bodies and a few polymorphs and lymphocytes. The lesion contained a mass of macrophages, many of them stuffed with L.D. bodies and some lymphocytes and plasma cells. The macrophages at the centre of the lesion were much richer in parasites than those at the periphery.

EXPERIMENTS ON MICE

Parrot and Donatien (1927) showed that cultures of *L. tropica* produce local lesions in mice when inoculated into the tail. We adopted the following method. Cultures of *L. tropica* are injected subcutaneously with a syringe into the tail until the upper half of the latter is distended. The distended part is then scarified at various points. In successful inoculations, lesions rich in L.D. bodies appear on all the scarified points. The L.D. bodies found in lesions in mice are much bigger than those produced by the same strain in man and may be almost as big as a red cell. L.D. bodies in large numbers are found eight to ten days after inoculation, but they may appear as early as the fifth day.

Twenty-six mice were inoculated with the dog strain from Baghdad.

Result. Negative during an observation period of six weeks to seven months.

The above result is in marked contrast to that obtained with a strain of canine visceral Leishmaniasis from Tunis presented by Professor C. Nicolle.

This strain nearly always produced local lesions in the tail of white mice. Out of twenty-four mice, only two did not take the infection. The incubation period varied from eight days to seven months. As in the case of *L. tropica* in mice, the L.D. bodies are very large. In the early stages of the infection the lesions are almost vesicular and contain a thick white fluid rich in macrophages and polymorphs stuffed with L.D. bodies. The polymorphs may be present in large numbers in spite of the absence of secondary infection. In the later stages the whole affected surface ulcerates and becomes covered with crusts (Pl. V, fig. 1). The ulceration may extend beyond the tail on to the back of the animal. There is no spontaneous cure and the lesions remain till the tail drops off.

A strain of Leishmania was isolated from a dog which had been infected with visceral Leishmaniasis by inoculation of bone marrow from a naturally infected child in Naples. Nine mice were inoculated into the tail with cultures of this strain.

Result. Negative during an observation period of one year.

Twelve mice were similarly inoculated with cultures of the human strain from Naples.

Result. Negative during an observation period of one year.

Thus in spite of the fact that naturally occurring visceral Leishmaniasis of the dog and the disease induced in dogs by inoculation from cases of human visceral Leishmaniasis are very similar, cultures from the two conditions may behave quite differently in mice.

The non-infectivity of the dog strain of cutaneous Leishmaniasis does not distinguish it from human cutaneous Leishmaniasis. Cultures of a strain of *L. tropica* isolated from a human being in Baghdad were inoculated into twenty mice with a negative result, whereas a strain which originated from a naturally infected *P. papatasii* in Jericho produced infections in mice almost without fail. During 1928 and 1929, over fifty mice were successfully inoculated from this strain. The lesions last a few months and then disappear spontaneously.

This Palestinian strain of *L. tropica* and the Tunis strain of canine visceral Leishmaniasis produce both visceral and cutaneous infections after intraperitoneal inoculation into mice. After intraperitoneal inoculation cutaneous lesions may appear anywhere but the neighbourhood of the anus and the vicinity of joints are favourite sites (Pl. IV, fig. 1). We have never observed visceral Leishmaniasis after inoculation into the tail in spite of the heavy local infection which followed.

The cultures isolated from Experiment I were found to be very slightly infective for mice.

15.12.29. Eight mice inoculated. Result negative up to 1.3.30.

19.1.30. Eight mice inoculated.

29.1.30. One mouse found positive. A single L.D. body was found in a smear.

24.2.30. The above mouse was negative but three others showed a slight infection. The infection rate was therefore 25 per

cent.   The strain was apparently modified by passing from the dog through man.

The most interesting results were obtained with a strain of South American Leishmaniasis presented by Professor E. Brumpt.

20.11.28.   Three mice inoculated from a culture.

3.12.28.   As no lesions appeared a second inoculation was given.

17.5.29.   One mouse died of unknown cause.

29.1.30.   One mouse was found to have ulcers near the root of the tail.   These were found to contain numerous L.D. bodies. As in the case of *L. tropica* and canine visceral Leishmaniasis, the L.D. bodies were very large.   An area of skin on the back was partially depilated and was ulcerating in various points (Pl. V, fig. 2).   The ulcerated parts were found to contain L.D. bodies in small numbers.

It is obvious that parasites from the inoculated site entered the blood stream and settled in the skin near the nape of the neck. Smears of the liver, spleen and bone marrow were negative.   These observations indicate that the South American strains can invade the blood stream without causing visceral Leishmaniasis and settle at a considerable distance from the site of inoculation.

Previous to this experiment, twelve mice were inoculated with this strain with a negative result, but the observation period only lasted a few months.

The behaviour of strains of Leishmania in mice deserves further study.   The differences in infectivity for mice between the two Baghdad strains, Palestinian and Algerian strains may be accidental or may be an expression of a constant biological difference.   A large number of Baghdad strains from man and dog must be examined before the matter can be settled.

We have described the behaviour of various strains of Leishmania in mice in order to show that inoculation experiments on animals must be interpreted very cautiously.   We cannot conclude that the two Baghdad strains (human and canine) and the Palestinian strain belong to different species because of the differences in infectivity for mice just as we cannot conclude without further evidence, that the Palestinian strain of *L. tropica* and the Tunis strain of visceral canine *Leishmania* are identical because they both produce cutaneous lesions in mice,

Histologically the Leishmanias of mice resemble those of man, but there are several points of interest. The parasitised macrophages of mice are very amoeboid and frequently send out long thin pseudopodia full of L.D. bodies round and between various tissue cells. Thus, in section muscle cells may be seen with long thin strips of protoplasm full of L.D. bodies applied to a large part of their periphery. Long pseudopodia full of L.D. bodies are often found between connective tissue cells. In this way, L.D. bodies are deposited in dense masses of fibrous tissue.

THE BEHAVIOUR OF THE STRAIN OF CUTANEOUS CANINE LEISHMANIASIS IN SANDFLIES

Table I shows that a canine strain from Baghdad behaves similarly to *L. tropica* in *P. papatasii*, but there is a tendency for the infections to die out after nine days and the infection rate is lower than in some Palestinian strains which give a 100 per cent. infection rate with the concentrations of flagellates used in the above experiments.

As in *L. tropica*, the flagellates which ascended the cardia attached themselves to the rhabdorium and in many cases the cardia was choked with flagellates. Sections of whole sandflies show that large numbers of parasites may enter the pharynx. Experiments carried out with the same strain after its isolation from the volunteer in Experiment 1 gave rather similar results.

The results recorded do not distinguish the strain from *L. tropica* for, as shown in a previous paper (1929), a human strain isolated in Baghdad showed even a lower infection rate for *P. papatasii*.

SUMMARY AND CONCLUSIONS

A human being was infected with cutaneous Leishmaniasis from a culture from a naturally occurring oriental sore of a dog from Baghdad.

It is, therefore, proved that there is a cutaneous Leishmaniasis common to man and dog and that *Leishmania tropica* is a naturally occurring parasite of the dog.

Strains of *L. tropica* vary in their infectivity for mice.

A human and a canine strain of *L. tropica* were found to be non-infective for mice.

TABLE I.

Date of experiment	Age of culture (days)	Number of parasites per average feed	Number of sandflies fed	Total number positive	Days after fed	Number of sandflies examined	Remarks
14.7.29	10	100	14	8	5	1	Cardia and stomach infected.
					7	2	One positive in cardia and stomach.
					8	11	Six positive in cardia and stomach.
25.9.29	7	300	7	2	6	5	All negative.
					7	2	Both positive in cardia and stomach.
26.9.29	8	700	12	3	3	1	Negative.
					5	3	Negative.
					6	1	Negative.
					8	4	Three positive in cardia and stomach
					9	1	Negative.
					10	2	Negative.
1.10.29	6	1,300	26	14	3	1	Positive in stomach only.
					4	8	Four positive, in two cardia and stomach infected, in two the stomach only.
					5	13	Eight positive in cardia and stomach.
					6	1	Positive in cardia and stomach.
					7	1	Negative.
					19	2	Negative.
2.10.29	7	1,600	17	7	3	2	Both positive. Many flagellates in stomach and few in cardia.
					4	4	One positive in stomach and cardia.
					5	1	Negative.
					8	3	Two positive, one in cardia and stomach, one in stomach only.
					9	2	Two positive in cardia and stomach.
					12	1	Negative.
					13	4	Negative.

Cultures from a human lesion produced by a canine strain of *L. tropica* were found to be slightly infective for mice.

Cultures of a Palestinian strain of *L. tropica* and a Tunis strain of canine visceral Leishmania were found to produce cutaneous lesions in mice after inoculation into the tail and visceral and cutaneous lesions after intraperitoneal inoculation.

The histopathology of a cutaneous lesion in man produced by cultures of a canine strain of *L. tropica* was studied.

The behaviour of the canine strain of *L. tropica* in sandflies was studied.

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PLATE IV

EXPLANATION OF PLATE IV

Fig. 1. Mouse infected with *L. tropica*. Ulcers round the anus and swellings round the joints, eight months after intra-peritoneal injection.

Fig. 2. Papules in a human being produced by inoculating culture of a canine strain of *L. tropica*. Natural size.

Fig. 3. Section through one of the papules shown in Fig. 2. Magnification × 30. (*a*) Infiltrated mass in corium ; (*b*) Fluid containing free L.D. bodies between (*a*) and the epidermis.
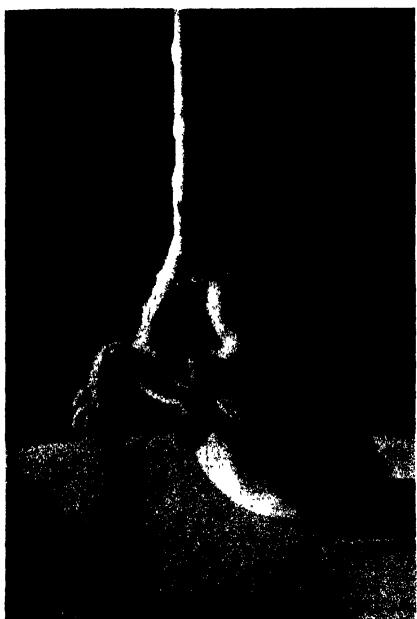
FIG. 1
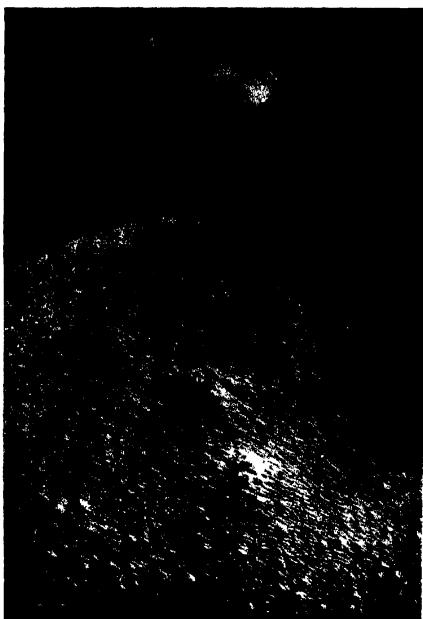
FIG. 2

FIG. 3.

EXPLANATION OF PLATE V

Fig. 1. Mouse with lesions on the tail, seven months after local inoculation of Tunis strain of *L. donovani* from a dog.

Fig. 2. Mouse infected locally with *L. brasiliensis*. Twelve months after infection. Note lesions on the tail and the partial depilation and ulcers on the back.
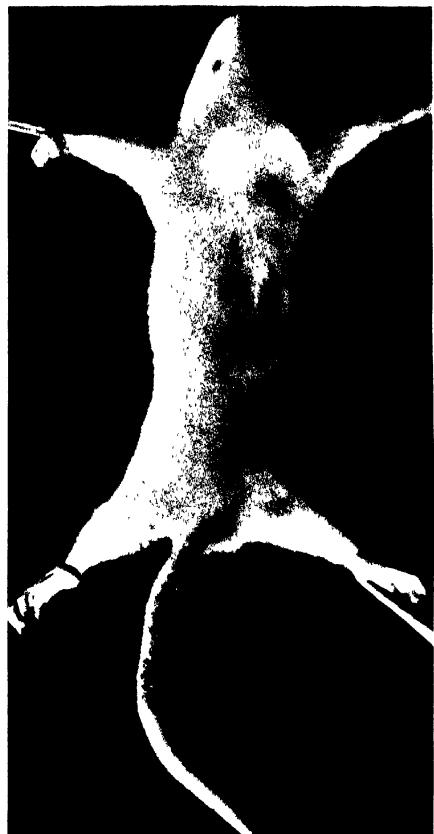
Fig. 1
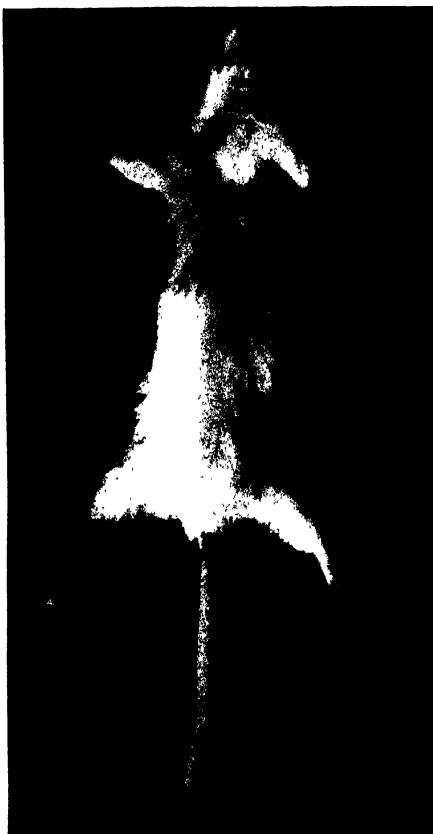
Fig. 2

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SOME ECOLOGICAL NOTES ON
GLOSSINA NEWSTEADI Austen*

BY

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WITH MAP

In June, 1928, I visited for the first time the lower reaches of the Lomami River, from its confluence with the Congo River at Isangi, as far as Obenge (see map), a distance of about 300 kilometres. Both on the up-river and the down-river journey, *G. palpalis* were captured in more or less large numbers, according to the different sections of the river, the flies becoming more numerous towards the source.

During the various halts, in the evening and sometimes in the morning, we caught a few *G. fusca* and *G. tabaniformis*. It may here be stated that in the whole district of Stanleyville (equatorial forests), *G. fusca* and *G. tabaniformis* are to be found everywhere in small numbers : this is in direct contrast with my experience in the many other districts in Tropical Africa I visited previously, in which these two flies are found in well defined and clearly separated areas.,

On reaching the mouth of the small River Elipa, I explored it in a canoe for about five kilometres, between 6 a.m. and 9 a.m., and captured fifteen tsetse flies, of which eight were *G. palpalis* and one was *G. tabaniformis*. Even at the moment of their capture, the remaining six flies attracted my attention by their appearance. They greatly resembled *G. pallicera*, but a superficial examination was sufficient to show that my flies were slightly larger and paler than true *G. pallicera*.

A few days later I explored by canoe another tributary of the Lomami, the Lobaye. I travelled sixty miles ; four days were taken in the up-river and two in the down-river journey. Numerous *G. palpalis* were captured. Three *G. fusca* and two *G. tabaniformis*

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were taken in the evening at various places where we stopped. As we travelled in several canoes, I was unable to examine the captured flies except at the stopping places, so that I knew neither the place nor the time of capture of the individual flies. I shall therefore limit myself to the statement that among the tsetse flies caught on the Lobaye I found nine specimens (4 ♂♂ and 5 ♀♀ belonging to the same species as that caught on the River Elipa, that is, the species resembling *G. pallicera.*

Later, on carefully re-examining the whole collection of flies captured during the voyage on the Lower Lomami, I again found two specimens of this same species, taken slightly above Litoko.

Major E. E. Austen, the well-known authority on *Glossina* of the British Museum, to whom I sent several specimens of this particular species, informed me that they belonged to a new species of the *G. palpalis* group. He later described it under the name *G. newsteadi,* Austen.

I had previously formed the opinion that *G. newsteadi* was confined exclusively to the Lower Lomami and particularly to certain of its tributaries, but on examining a small collection of tsetse flies from the River Aruwimi, taken by Dr. Franconi, between Hanalia and Yambuya, in May, 1929, I found eleven *G. palpalis* and two *G. newsteadi.*

As I was dealing with a new and probably quite local species, I wished not only to procure new material but also to study the particular habits of this tsetse and the area frequented by it. I therefore decided to return to the Lower Lomami at the first opportunity. This I have been able to do and I have just spent a month on this work (October, 1929). It will be seen that unfortunately the result of my recent researches is disappointing, probably owing to the fact that my journey was made in the bad season. The month of October was very wet and for two-thirds of the time the rain kept me in my tent or under cover. The banks of the Lomami and of those of its tributaries visited by me were flooded ; it was, therefore, useless to look for pupae, even of *G. palpalis* which, in normal conditions are easily found. In view of the fact that I am studying a new and quite unknown species, and that it is likely that I shall not return to the district for a long time, I think that my preliminary observations will be of some interest.

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GL. NEWSTEADI
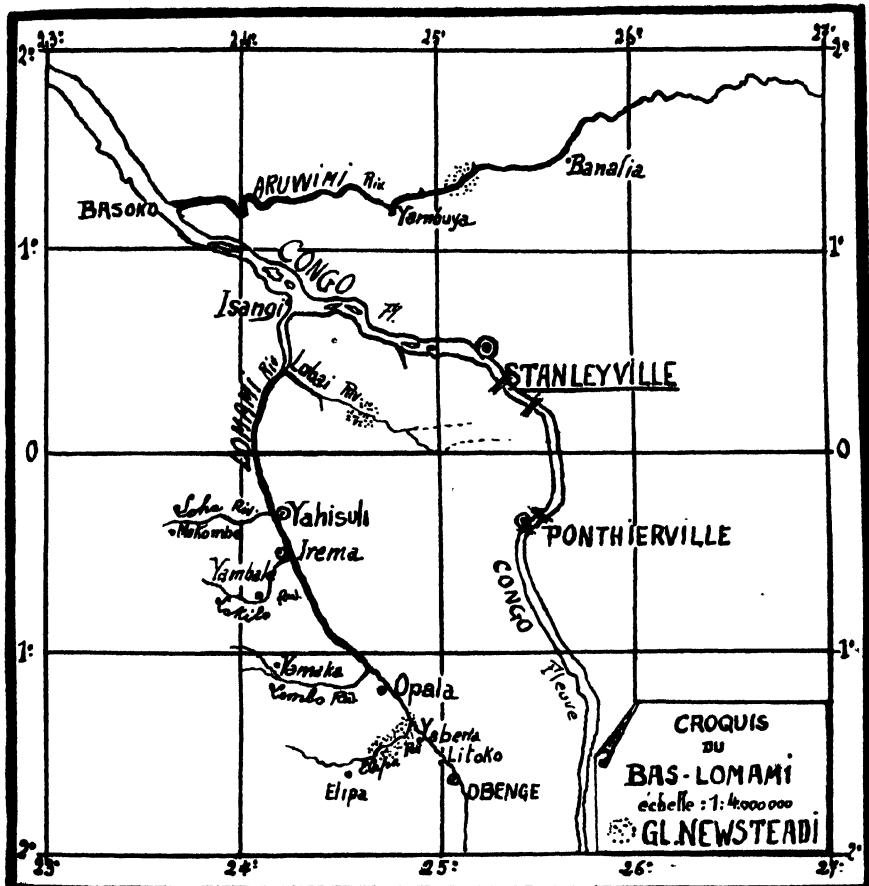
In June, 1928, I explored the River Lomami by steamer as far as Obenge, and the River Lobaye by canoe. I have mentioned above the tsetse material I found, and that at the mouth of the small river, Elipa (at Yabena), I had found a considerable number of *G. newsteadi*. I therefore wished to examine it more carefully. But as the Lower Lomami has several small tributaries which are navigable by canoes, I decided to study them also. This I did in October, 1929.

These small rivers are :—

1. The Loha, between Mokombe and Yahisuli ; about 70 kilometres.
2. The Lokilo, between Yambale and Irema ; about 30 kilometres.
3. The Lombo, between Yemaka and Opala ; about 35 kilometres.
4. The Elipa, between Elipa and Yabena ; about 30 kilometres.

On the first three rivers, I found *G. palpalis* and a few *G. fusca*, *G. tabaniformis*, but not a single *G. newsteadi*. On the Elipa, however, I not only captured a certain number of *G. newsteadi*, but I was able to make some ethological observations on this fly.

A curious fact is that in October, 1929, I found, in general, fewer tsetse flies than in June, 1928. This was particularly noticeable at the mouth of the River Elipa, which I had examined at these two different dates. The natives of the river banks, especially the paddlers of my canoes, assured me that at flood-time, that is in the rainy season, tsetse flies are much rarer than when the water is low. In the non-equatorial regions, the contrary is more usual, but in the district under discussion, which lies between 1° N. and 2° S., it appears that the flooding of the river either diminishes the number of the flies or causes them to leave the river banks.

On account of the heavy rains, I was able to work for five days only out of the ten I spent on the Elipa. In the upper reaches of the river, tsetse flies were extremely rare, *G. palpalis* only being found, whilst in the lower reaches, towards its confluence with the Lomami, they were less rare and more varied. It must be here noted that the river vegetation has no influence on this difference in numbers and species, as the whole district, whether along or between the rivers, is covered with uninterrupted equatorial forest.

Among the eighty-two flies captured on the Elipa and on the banks of the Lomami near the mouth of the former, at Yabema, were the following :—

(1) 44 *G. palpalis*, 27 ♂, 17 ♀   = 53·5 per cent.

(2) 8 *G. fusca*, 2 ♂, 6 ♀   = 10   ,,

(3) 5 *G. tabaniformis*, 4 ♂, 1 ♀ = 6   ,,

(4) 25 *G. newsteadi*, 10 ♂, 15 ♀ = 30·5 ,,

It may, therefore, be seen that of the four species of *Glossina* captured between the Elipa and Yabena, *G. newsteadi* is the second most numerous and is in the proportion of 57 per cent. to *G. palpalis*. I must, however, state that this calculation is purely arbitrary, since it depends on the time and place of capture. The following observations will prove this fact :—

1. We left Elipa at 9 a.m., and we travelled until 1 p.m. (20 kms.). Following on heavy night rains, the morning was very cool, and the sun did not appear until 10 p.m. We saw very few Glossina in the canoe. On this journey we captured ten flies altogether, of which eight were *G. palpalis* and two *G. newsteadi*, the latter being caught between 9 a.m. and 10 a.m.

We pitched our camp in the forest on the bank of the river. No tsetse were seen during the afternoon, which was overcast and cool, but after sunset one *G. tabaniformis* and two *G. newsteadi* were taken near the camp and at a certain distance from the river. At 7.30 p.m., well after nightfall, one *G. fusca* was caught near the camp.

2. On the next day we started at 7 a.m. and reached the mouth of the Elipa at 9 a.m. The sky was much overcast and it was very cool. Only four tsetse were captured—one *G. palpalis* and three *G. newsteadi*.

3. We paddled four miles up the Elipa, starting at 3 p.m. Between 3 p.m. and 5 p.m. we caught five *G. palpalis*. On the way back, after 5 p.m., we took two *G. newsteadi*. The same evening my hunter brought me three tsetse caught in the forest before sunset—one *G. fusca*, one *G. tabaniformis*, and one *G. newsteadi*.

4. The next day we made the same journey up the Elipa, between 9 a.m. and mid-day. The day was rather warm. We captured five *G. palpalis* but no other tsetse flies. At 2 p.m. my '.fly-boys' went along a path on the bank of the Lomami, and at 3 p.m. they brought back five *G. palpalis* and one *G. newsteadi*. They repeated

this expedition at about 6 p.m. (sunset), and brought back one *G. fusca* and one *G. newsteadi.*

5. The following day we walked between 2 p.m. and 3 p.m., along the Lomami, opposite the confluence of the Elipa. We captured six *G. palpalis* and one *G. fusca.* But at about 6 p.m. (sunset), two *G. palpalis* and four *G. newsteadi* were caught on the bank of the Lomami, not far from the mouth of the Elipa

SUMMARY OF OBSERVATIONS

From the above observations it would appear that from the ecological point of view, *G. newsteadi* occupies an intermediary place between the *G. palpalis* group and the *G. fusca* group, whilst if *G. newsteadi* occurs normally with *G. palpalis* on the banks of the rivers, it is also found with *G. fusca* and *G. tabaniformis*, at a distance from water. And although sometimes *G. newsteadi* is captured in broad daylight, it is active chiefly in the morning and the evening, which also makes it akin to the *G. fusca* group.

On the other hand, although from a morphological point of view, *G. newsteadi* belongs to the *G. palpalis* group, it is distinguished from most species in this group by its greater size and by its lighter colour, which allies it to the *G. fusca* group.*

We therefore see that the biological, or at any rate, the ecological differences correspond to the morphological ones.

I am unable to give any details on the feeding habits of this new species, as I was never bitten by any tsetse fly on the Elipa.

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Austen, E. E. (1929). A new Tsetse-fly of the *Glossina palpalis* group occurring in the Belgian Congo. *Bull. Ent. Res.*, **20**, 1-4.

*The author has requested the Editors to add the following communication from Major E. E. Austen, D.S.O.:—"In pinned specimens, at any rate, no such difference is apparent. The museum examples of *G. newsteadi* are just as dark as those of *G. caliginea*, and darker than those of *G. pallicera*. Moreover there is certainly no constant difference in size. (8.1.30.)"

CONTRIBUTION À L'ÉTUDE DES TRYPANOSOMIASES DES SUIDÉS

PAR

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PLATES VI, VII

INTRODUCTION

De toutes les trypanosomiases animales, celles des suidés sont certainement les moins bien connues. Certaines espèces de trypanosomes furent trouvées *naturellement* chez les cochons sauvages et domestiques ; la pathogénicité d'autres trypanosomes pour les cochons fut prouvée expérimentalement.

1. *Tr. brucei* fut trouvé très pathogène pour les cochons par des expériences de transmission. Autant que nous sachions par la littérature se trouvant à notre disposition, ce trypanosome n'a jamais été trouvé chez le cochon en infection naturelle. Mais *Tr. suis*, trouvé chez le porc et chez le phacochère dans l'Est africain, est plus que probablement une variété, ou même une synonymie, de *Tr. brucei*.

2. Le trypanosome le plus commun des suidés, aussi bien domestiques que sauvages, est certes *Tr. congolense*. Sa pathogénicité est variable suivant les souches et même, peut-être, suivant les variétés de ce groupe de trypanosome.

3. En 1914, Bruce et collaborateurs, ont trouvé et décrit une nouvelle espèce de trypanosome, très pathogène pour le porc : *Tr. simiae*. Ce trypanosome affecte le singe, les ovidés, les capridés et les suidés, mais non pas les bovidés. Il fut également trouvé, dans le Nyassaland, chez le phacochère. Ce qui caractérise biologiquement *Tr. simiae*, c'est son extrême virulence pour le singe et pour le porc.

S'il n'est pas difficile de distinguer *Tr. brucei* de *Tr. congolense*, la détermination de *Tr. simiae* n'est pas bien aisée. D'après Bruce et collaborateurs, *Tr. simiae* appartient au groupe de *Tr. pecorum*

(= *Tr. congolense*). Mais il s'en distingue par sa plus grande taille (18μ de long, en moyenne, au lieu de 14μ chez *Tr. congolense*). Comme c'est surtout l'absence d'un flagelle libre qui caractérise le groupe congolense, voici ce que disent à ce sujet les auteurs précités concernant *Tr. simiae ;*

' Il est difficile de dire si cette espèce a un flagelle libre ou non. Dans des préparations soigneusement colorées et avec un bon éclairage, il semble que dans la plupart des cas la membrane ondulante s'étend jusqu'au bout du flagelle. Toutefois, dans la plupart des préparations, les derniers deux ou trois microns du flagelle semblent être souvent libres.'

Nous ajouterons que, d'après Hornby, il est possible que *Tr. simiae* n'est qu'une ' race ' de *Tr. congolense*.

En ce qui concerne le Congo Belge les suidés ne figurent, pour ainsi dire, pas dans les nombreux et intéressants travaux de Dutton et Todd, de Broden et de Rhodain sur les trypanosomiases animales de ce vaste pays. Toutefois, parmi les animaux domestiques d' Elisabethville (Katanga), examinés par Rodhain et collaborateurs en 1912, 7 porcs sur 11 furent trouvés infectés de *Tr. congolense*.

En 1917, Greggio signale une forte infection à *Tr. congolense* des porcs indigènes de la vallée de l'Inkissi (Bas-Congo). Il ajoute que cette trypanosomiase semble être très peu pathogène.

4. En 1922, Walravens trouve au Katanga un trypanosome très pathogène pour le porc et qu'il décrit sous le nom de *Tr. rodhaini*. Il s'agit d'un trypanosome monomorphe, long et mince, avec une membrane ondulante peu prononcée, avec un blépharoplaste marginal et un *flagelle libre*. Outre le porc, ce n'est que le singe (cercopithèque) qui s'est montré réceptif à ce trypanosome. Le cobaye, le lapin et le bœuf sont réfractaires.

Nous ajouterons que Hornby signale avoir trouvé chez le porc un trypanosome qui lui semble être le même que celui décrit par Walravens et qu'il a identifié avec *Tr. uniforme*. D'autre part, nous trouvons dans le traité de C. M. Wenyon (p. 1560) la remarque suivante concernant le trypanosome trouvé par Walravens :

'Walravens a donné le nom de *Tr. rodhaini* à un trypanosome trouvé dans le cochon au Congo Belge. Il ressemble à *Tr. vivax* par son flagelle libre mais il s'en distingue par ses mouvements moins actifs et par son corps étroit. Comme les mensurations ne sont pas

indiquées, il est évident que de nouvelles investigations sont nécessaires avant de pouvoir accepter la validité de cette espèce.'

Depuis lors Walravens a donné les mensurations de son trypanosome. Nous y reviendrons plus loin. Mais avant cela nous voulons passer au sujet principal de cette étude, à nos observations et constatations personnelles.

En parlant de la trypanosomiase en Afrique, on ne peut passer sous silence sa cause principale—les tsétsé. D'autre part, il serait déplacé d'entrer ici dans de longues considérations à ce sujet. Nous nous bornerons donc à quelques renseignements et remarques qui concernent directement notre sujet.

On connait le role des glossinés dans la transmission de *Tr. congolense* et de *Tr. vivax*. Bruce et collaborateurs incriminent *Gl. morsitans* dans la transmission de *Tr. simiae*. Près d'Elisabethville, où Walravens a trouvé *Tr. rohdaini*, il n'existe actuellement plus de tsétsé : ni *Gl. morsitans*, qui y existaient précédemment, ni *Gl. palpalis*.

En ce qui concerne les environs de Stanleyville, il s'agit de la forêt équatoriale où il n'existe pas de *Gl. morsitans*. Nous y avons par contre trouvé trois autres espèces : *Gl. palpalis*, *Gl. fusca* et *Gl. tabaniformis*. Mais toutes ces trois espèces sont si rares dans les environs plus ou moins immédiats de Stanleyville, et partant autour des quelques fermes, que les poussées de trypanosomiase chez les bovidés que nous avons pu observer dans plusieurs troupeaux, sont difficilement explicables sans l'admission du rôle transmetteur des autres mouches hématophages. Il en est probablement de même en ce qui concerne la trypanosomiase des cochons.

A Ibembo (Uélé), on trouve les mêmes trois espèces de glossines qu'à Stanleyville.

Ici, dans cette étude, nous ne nous occupons que des trypanosomiases des suidés. Nous nous bornerons par conséquent à une simple énumération des espèces de trypanosomes trouvées chez les bovidés et les ovidés de Stanleyville et de l'Uélé.

BOVIDÉS.

1. *Tr. congolense* (le plus fréquent).
2. *Tr. uniforme* (moins fréquent).
3. *Tr. vivax* (assez rare).
4. *Tr. brucei* ? Peut-être.

OVIDÉS.

1. *Tr. congolense* (le plus fréquent).
2. *Tr. vivax* (assez fréquent).
3. *Tr. brucei* (rarissimes).
4. *Tr. uniforme* ? Peut-être.

Observations personnelles sur les trypanosomiases des suidés de Stanleyville et de quelques autres localités de la Province Orientale du Congo Belge.

No. d'ordre	Localité et nom des troupeaux	Nombre de cochons examinés	Nombre de trypanosés	Espèce de trypanosome	Observations
A. Stanleyville					
(1)	Ile Barmann ...	7	1	*Tr. congolense*	
(2)	Kibibi	10	0	—	Verminose intestinale et bronchiale
(3)	Km 10	10	0	—	Idem
(4)	Mission catholique	65	? 7	?	
(5)	Tshopo	356	? 6	?	
(6)	Mathot	76	? 7	?	
	Total ...	523			
B.	Ponthierville ...	8	0	—	
C. Uélé :					
(1)	Niangara	39	0	—	
(2)	Ibembo	40	?	?	
	Total ...	87			
	Et en plus :	cochons sauvages (rouges)			
(1)	Environs de Stanleyville	3	0	—	
(2)	Bas-Lomami ...	3	1	*Tr. congolense*	

Le tableau tel quel semble évidemment bien étrange. Non seulement à part deux rubriques, il ne spécifie pas l'espèce de trypanosome trouvé, mais il n'indique même pas le nombre exact des bêtes

trouvées infectées. Mais on va voir pourquoi nous n'avons pu donner un tableau plus précis des trois grands troupeaux de suidés de Stanleyville et de celui d'Ibembo.

I. IBEMBO (Uélé)

1.—En 1928, nous avons reçu du Docteur Henneaux d'Ibembo (Uélé) un frottis de sang coloré et provenant d'un cochon de cette localité. Les frottis avaient l'air d'une culture de trypanosomes. Malgré les très nombreux trypanosomes en division et la mauvaise coloration du frottis, on pouvait voir qu'il s'agissait d'un trypanosome spécial : long et mince avec un petit flagelle libre et le blépharoplaste plutôt latéral. Bref, ce trypanosome nous rappelait celui trouvé par Walravens chez quelques cochons du Katanga (Elisabethville) et décrit par lui sous le nom de *Tr. rodhaini*.

Nous demandions donc au Docteur Henneaux de nous envoyer du nouveau matériel en bon état et, si possible, un cochon infecté vivant. Il nous fut d'abord répondu qu'on ne trouvait plus de trypanosomes chez les cochons en question, ceux-ci ayant reçu entretemps des injections d'atoxyl (? !). Plus tard, nous reçûmes un petit cochon vivant, mais durant deux mois nous n'avons jamais rien trouvé dans son sang. Notre collaborateur, le vétérinaire Storck, fut alors chargé de se rendre à Ibembo pour examiner les cochons et pour apporter du matériel. Mais aucun des cochons (au nombre de 40) ne fut trouvé trypanosé à l'examen de leur sang à frais. Les gouttes épaisses et les frottis étalés, examinés ensuite au Laboratoire de Stanleyville, furent également trouvés complètement négatifs. N'empêche que de plusieurs frottis envoyés peu après par le Docteur Henneaux, et provenant de plusieurs cochons du même troupeau, ceux de deux cochons furent trouvés positifs. Les trypanosomes de ces deux porcs sont reproduits dans la figure II : A et B. On constatera, en examinant cette double figure, qu'il ne s'agit pas tout à fait du même trypanosome. Tandis que dans ' B ' de la figure il s'agit d'un trypanosome monomorphe, il s'agit d'un trypanosome polymorphe, ou d'une infection mixte, dans ' A ' de la même figure.

Nous y reviendrons tout à l'heure, après avoir exposé la situation plutôt bizarre de la trypanosomiase des cochons de Stanleyville.

II. STANLEYVILLE

A notre arrivée à Stanleyville en 1927, il y avait deux grands troupeaux de cochons : à la mission catholique et à la ferme de la Tshopo. Les bêtes de ces deux troupeaux se portant bien, nous ne les avons pas examinées. Par contre, les cochons de la ferme de la Kibibi et de celle du Km 10 dépérissaient. Nous n'avons pas trouvé de trypanosomes chez ces bêtes. La cause de leur dépérissement était une forte verminose (strongyles) intestinale et même bronchiale.

Au début de 1928, un colon installe, sur une petite ile du Fleuve Congo, tout près de Stanleyville, un troupeau de petit bétail : chèvres, moutons et cochons. Une forte mortalité s'étant déclarée parmi ces derniers, nous examinons, en juin, les sept cochons restants. Un seul est trouvé atteint de *Tr. congolense* (typique). Nous le gardons en observation pendant une huitaine de jours. Les trypanosomes sont tantôt bien rares et tantôt (suivant les jours) assez nombreux. Bien malade au début, le petit cochon semble se remettre peu à peu (sans traitement). Il est abattu.

1. Troupeau de la Mission Catholique (Fig. III).

Entre le 27 et le 30 mars 1929, deux cochons y meurent presque subitement. L'examen du sang du cœur (prélevé post mortem) révèle la présence de trypanosomes qui nous semblent à ce moment-là appartenir au groupe Congolense. Le 3 juin, on nous apporte au Laboratoire un cochon in extremis (cochon No. 3). Son sang grouille de trypanosomes dont la plupart sont en division. A frais, on constate des mouvements sur place. A l'examen des frottis étalés, on voit qu'il s'agit d'un trypanosome spécial, long et mince, avec un petit flagelle libre et le blépharoplaste marginal. (Fig. III B) Nous injectons avec le sang de ce cochon un mouton et un cobaye. (Voir plus loin : Essai de transmission No. 1.)

Nous décidons alors d'examiner tout le troupeau.

Sur 54 cochons examinés, le 4 juin, nous trouvons un seul trypanosé. L'animal est abattu. Le seul frottis fut examiné pendant notre absence et égaré ensuite. Nous ne pouvons donc pas donner de renseignements sur le trypanosome trouvé.

Depuis lors, tout va bien jusqu'au 6 septembre. Mais à cette

date-là un cochon meurt de nouveau et nous trouvons dans le sang du cœur (post mortem) d'assez nombreux trypanosomes.   Il s'agit d'un trypanosome ' long et mince ' ; mais comme le sang a été prélevé le lendemain de la mort de l'animal, les flagellés sont dans un mauvais état et il est difficile de dire de quelle espèce de trypanosome il s'agit.   Nous décidons alors de réexaminer tout le troupeau.

Sur 65 cochons examinés, le 11 septembre, nous trouvons deux infectés.   Chez un d'eux (No. 65) les trypanosomes sont très nombreux (voir figure III A).   Les deux animaux sont abattus.

2. Troupeau de la ferme de la Tshopo.

En mai 1929, une assez forte mortalité se déclare parmi les cochons de cette ferme.   Entre le 18 et le 31 mai, 5 cochons meurent presque subitement.   A l'autopsie de toutes ces cinq bêtes nous trouvons des trypanosomes dans le sang cardiaque.   Dans tous ces cas, il s'agit plus ou moins du même trypanosome spécial dont nous avons déjà parlé plus haut : long, mince, avec un blépharoplaste latéral et un petit flagelle libre.   La mensuration d'un certain nombre de trypanosomes encore bien conservés donne les chiffres moyens suivants :

(a) Longueur du trypanosome, flagelle non compris, 15 μ.

(b) Longueur du flagelle, 6 μ.

(c) Largeur du trypanosome, 1·6 μ.

(d) Distance du blépharoplaste à l'extrémité postérieure, 0.7 μ.

Mais le sang étant prélevé le lendemain de la mort de ces bêtes, les trypanosomes sont, en général, en mauvais état et difficilement déterminables.   Quelques trypanosomes trouvés dans le sang du cœur d'un de ces cochons de la Tshopo sont reproduits dans la figure III c.

Nous tâchons, bien entendu, d'obtenir un cochon malade (in vivo), mais nous n'y parvenons pas.   Le fermier nous déclare que la maladie est de trop courte durée.   Encore en bon état le matin, l'animal cesse de manger l'après-midi, se couche le soir et meurt dans la nuit.

Nous décidons alors d'examiner tout le troupeau de cette ferme. Les 356 cochons de la Tshopo sont examinés entre le 1 et le 11 juin.   *Pas une seule bête n'est trouvée trypanosée.*

Mais des morts quasi subites continuent. Et entre le 11 juin et le 28 septembre, 5 autres cochons meurent. Mais chez ces cinq bêtes nous ne trouvons pas de trypanosomes, ni d'ailleurs rien d'autres. Pour pouvoir tirer la chose au clair, c'est-à-dire pour pouvoir tomber sur notre trypanosome, encore in vivo, nous décidons de réexaminer le troupeau. C'est ce que nous faisons en octobre. Mais après avoir réexaminé 173 cochons—et les plus suspects—sans le moindre résultat, c'est-à-dire sans avoir trouvé de trypanosomes, nous renonçons à examiner les autres, qui se portaient d'ailleurs tous très bien. Mais entre le 28 septembre et le 25 novembre, six autres cochons meurent, bien rapidement, comme d'habitude. Nous avons la chance de pouvoir examiner une de ces bêtes in vivo, bien in extremis, mais nous ne trouvons rien chez elle. Quant aux cinq autres cochons, examinés post mortem, nous n'avons trouvé de trypanosomes qu'une seule fois, dans le sang cardiaque.

3.—Troupeau du colon M.

Entretemps, fin 1928, un colon installé à 5 kms de Stanleyville, fait venir dans sa ferme un certain nombre de cochons, dont une partie de la ferme de la Tshopo.

Entre le 28 juin et le 13 octobre 1929, 7 cochons meurent de la manière habituelle, c'est à-dire très rapidement. Tous sont autopsiés. Chez trois cochons nous ne trouvons rien ; chez les quatre autres nous trouvons des trypanosomes dans le sang-suc cardiaque. Autant que l'on peut juger, il s'agit toujours de notre trypanosome spécial.

Nous décidons alors d'examiner tout le troupeau de cette ferme. Mais sur 76 cochons examinés, le 14 octobre, pas un seul n'est trouvé trypanosé !

Le 15 décembre, on nous signale un cochon mort et deux malades. L'autopsie du mort révèle une pneumonie et des trypanosomes dans le sang cardiaque. Quant aux deux vivants, nous trouvons des trypanosomes chez tous les deux : de nombreux chez un (cochon noir) et de rares, chez l'autre (cochon rouge). Le lendemain, 16 décembre, le cochon noir meurt (fig. IV A). Le cochon rouge est très malade et les trypanosomes sont déja très nombreux (fig. IV B). A frais les trypanosomes ne sortent pas du champ microscopique. Nous injectons du sang du cochon rouge : à un

petit cochon, à un jeune mouton et à deux cobayes (Voir : Essai de
transmission No. 2).   Il (le cochon rouge) meurt le lendemain,
le 17 décembre.

* * * * *

A deux reprises nous avons donc réussi, après tant de difficultés,
il est vrai, à dénicher notre trypanosome chez des cochons vivants
et à le transmettre, ou du moins à essayer de la transmettre, à des
animaux neufs : une fois dans le troupeau de la mission et une
autre fois dans le troupeau du colon M.

Voyons à présent le résultat de ces deux essais de transmission :

Premier essai. Troupeau de la Mission.

Cochon No. 3, in extremis, 3 juin 1929.   Nombreux trypano-
somes (fig. III B).

Injection de sang à un cobaye et à un mouton.   Le cobaye
reste négatif jusqu'au 10 juillet, soit pendant 37 jours.

Quant au mouton, les trypanosômes apparaissent dans son
sang le 20 juin, soit 17 jours après la transmission, mais ils
disparaissent bien vite, puisque l'on n'en trouve plus le 24 juin,
quatre jours plus tard.   Et le mouton reste négatif jusqu'au
10 juillet, date de sa mort de cause inexpliquée.

Quant à l'espèce du trypanosome trouvé, il s'agit, comme on
peut le constater par la figure V A, d'un trypanosome du groupe
Congolense mais très bizarre (*Tr. montgomeryi* ?).   A frais, il se
mouvait sur place.

Deuxième essai. Troupeau du colon M.

Cochon rouge.   In extremis, le 17 décembre 1929.   Nombreux
trypanosomes (fig. IV B).   On injecte de son sang à :

(1) Un petit cochon.   } Préalablement évidemment examinés, au
(2) Un jeune mouton.   } point de vue de la trypanosomiase.
(3) Deux cobayes.   }

Tous les quatre animaux sont examinés journellement : à frais,
en goutte épaisse et en frottis.   Et voici le résultat :

(1) Les deux cobayes restent négatifs jusqu'au 15 janvier 1930,
soit durant un mois.

(2) Chez le petit cochon les trypanosomes apparaissent le 4ème
jour après la transmission, soit le 21 décembre.   A frais, les trypano-
somes se meuvent sur place.   Ils sont relativement rares et on voit

(dans les gouttes épaisses et dans les frottis) des formes de division. Durant cinq jours consécutifs (du 21 au 25.12) nous voyons le même trypanosome (fig. IV D et E) qui est toujours plutôt rare. Mais dans la nuit du 25 au 26 décembre, notre cochon d'expérience meurt subitement. Sauf une dégénérescence graisseuse du foie on ne trouve rien d'anormal à l'autopsie. Chose bizarre : nous ne trouvons pas de trypanosomes dans le sang-suc du cœur (prélevé post mortem).

(3) Chez le mouton, les trypanosomes apparaissent également le 4ème jour après la transmission : le 21 décembre. Nous retrouvons les trypanosomes les quatre jours consécutifs, du 21 au 25 décembre. Ils sont toujours très rares, si rares que nous ne les trouvons pas à l'examen du sang à frais. A deux reprises seulement nous les trouvons dans les frottis étalés (fig. V B et C). Les autres jours nous ne les décelons que dans les gouttes épaisses.

Mais à partir du 26 décembre (et jusqu'au 15 janvier) nous n'avons plus trouvé de trypanosomes chez notre mouton.

DISCUSSION

Ce qui caractérise cliniquement notre trypanosomiase des cochons de Stanleyville (et, d'après les renseignements obtenus, celle d'Ibembo également), c'est sa faible contagiosité, d'abord, et sa grande acuité, ensuite. Notre trypanosome a l'air d'être extrêmement virulent pour le porc qu'il tue en quelques jours. La marche de la maladie est si aiguë qu'il n'existe pas de prodromes. Suraiguë et fatale : telle est la définition de cette trypanosomiase porcine. Mais, d'autre part, ce trypanosome semble être peu contagieux, puisque dans les troupeaux infectés nous n'avons constaté durant presque deux années que des cas sporadiques.

A notre arrivée à Elisabethville, au début de 1925, un grand troupeau de porcs d'une ferme voisine (Km 17) venait d'être littéralement décimée par une épidémie trypanosomique. C'est justement de la ferme en question que provient le trypanosome signalé et décrit par Walravens. A nos premières constatations de cas de trypanosomiase aiguë parmi les cochons de Stanleyville, et surtout après avoir constaté qu'il s'agissait du même trypanosome qu'à Elisabethville, nous nous attendions à voir une très forte

mortalité. Or, la morbidité et la mortalité sont restées ici tout à fait sporadiques. Dans ces conditions, nous n'avons pu essayer aucun traitement.

La trypanosomiase habituelle du porc, provoquée par *Tr. congolense*, est caractérisée par sa plus ou moins grande chronicité. Nous faisons abstraction de la constatation de Greggio, qui concerne le porc indigène qu'il faut considérer comme une espèce de gibier. De même que les chiens indigènes résistent dans les régions à *Gl. morsitans*, de même le porc indigène possède une résistance spéciale, une accoutumance (dans le cas de Greggio) à la *Gl. palpalis* et au *Tr. congolense*. Mais même chez le porc importé, *Tr. congolense* provoque une maladie chronique ou, du moins, sub-aigüe. En ce qui concerne les trois autres espèces de trypanosomes pathogènes pour le cochon et trouvées chez lui en infection naturelle : *Tr. suis = Tr. brucei*, *Tr. simiae* et *Tr. rodhaini*, toutes les trois semblent provoquer une infection très aigüe, comme dans les cas constatés par nous. A laquelle de ces trois espèces notre trypanosome de Stanleyville (et d'Ibembo) appartient-il ? Et puis, le trypanosome de Walravens décrit par lui sous le nom de *Tr. rodhaini* est-il vraiment une nouvelle espèce, biologiquement ou du moins morphologiquement distincte des autres espèces connues, ou s'agit-il d'une espèce déja connue : par exemple de *Tr. uniforme*, comme le croit Hornby, de *Tr. vivax* comme le supposait éventuellement Wenyon, ou même de *Tr. simiae ?* C'est ce que nous allons tâcher de tirer au clair.

Tr. brucei est trop connu pour devoir s'y arrèter. La description de *Tr. simiae* est également connue et la spécificité de ce trypanosome semble être admise. En ce qui concerne *Tr. rodhaini*, nous ne nous sommes pas bornés à consulter les quatre notes publiées par Walravens, mais nous avons demandé à ce dernier de nous envoyer quelques frottis de son trypanosome. Notre distingué confrère et ami nous envoya aimablement deux frottis : un de sang de cochon et un autre de sang de singe-cercopithèque, infecté avec du sang de porc. Nous fîmes dessiner les trypanosomes de ces deux frottis (fig. I A et E) à la même échelle que les trypanosomes trouvés par nous à Stanleyville (et à Ibembo). Nous avons donc ainsi un point de comparaison morphologique très important.

Les belles figures dessinées par notre assistante Mademoiselle

Droeshaut simplifieront et faciliteront de beaucoup notre tâche. Elles permettront en outre à d'autres de se faire de visu une idée exacte de la question : d'autant plus qu'aux figures nous joignons les originaux, c'est-à-dire les frottis ayant servi à dessiner les diverses figures. Nos divers trypanosomes (et ceux de Walravens) furent dessinés à l'échelle approximative de × 1600. Il suffit alors de comparer ces figures à celles de *Tr. simiae* (× 2000) se trouvant dans le travail original de Bruce et collaborateurs et reproduites dans le traité de Wenyon.

Mais avant tout rappelons brièvement la description de *Tr. simiae* et de *Tr. rodhaini*, telle qu'elle est donnée par leurs auteurs respectifs : Bruce et Walravens.

I. *Tr. simiae*. Corps allongé, étroit, ondulé. Extrémité postérieure arrondie ou légèrement pointue. Noyau oval. Blépharoplaste petit, rond, situé à 1·5 μ environ de l'extrémité postérieure et placé latéralement. Membrane ondulante fortement plissée. Flagelle ne dépasse pas fréquemment la membrane ondulante ; quelquefois extrémité libre de 1 ou 2 μ. Longueur moyenne : 17 à 18 μ. Largeur moyenne : 1·75 μ. A frais, mouvements vifs sur place : quelques individus traversent le champ microscopique.' (Bruce).

II. ' *Tr. rodhaini*.—Corps allongé à noyau situé plus près de l'extrémité antérieure que de l'extrémité postérieure. Blépharoplaste situé à une très petite distance de l'extrémité postérieure et toujours accolé à la paroi. Membrane ondulante très légèrement accusée. Flagelle libre assez long. Toujours monomorphe. Longueur moyenne du trypanosome, flagelle compris : 19·8 μ. Longueur moyenne du flagelle : 7 μ. Largeur du trypanosome : 0·7-0·9 μ. A frais, mouvements faibles, sur place.' (Walravens).

N.B. Walravens n'a, malheureusement, pas donné une seule description complète de son trypanosome. Les renseignements ci-dessus furent trouvés par nous dans plusieurs notes successives. C'est ainsi que dans une note Walravens situe le noyau dans la partie moyenne et dans une autre, plus près de l'extrémité antérieure.

Quelle est donc la différence entre ces deux trypanosomes, d'après la description de leurs auteurs ?

(1) *La largeur* qui est de 1·75 μ chez *Tr. simiae* et de 0·7 à 0·9, chez *Tr. rodhaini*.

(2) *Le flagelle libre* qui est de 7 μ chez *Tr. rodhaini* et seulement de 1 à 2 μ, ou même complètement absent, chez *Tr. simiae.*

(3) *La membrane ondulante* qui est fortement plissée dans *Tr. simiae* et peu marquée dans *Tr. rodhaini.*

Il est à noter que dans les deux trypanosomes le blépharoplaste est accolé à la paroi, c'est-à-dire qu'il est latéral, ou marginal (comme dans le groupe Congolense), et non pas terminal (comme dans le groupe Vivax). Et à ce propos nous nous permettons d'exprimer notre étonnement du peu d'importance que les auteurs attachent à la position du blépharoplaste dont la position latérale est pourtant si caractéristique pour le groupe Congolense, non moins caractéristique que sa position terminale, dans le groupe Vivax.

Certes, on peut m'objecter que la position du blépharoplaste n'est pas un signe absolu, que quelquefois il est difficile de dire si le blépharoplaste est sub-terminal ou sub-latéral. Mais il en est de même avec le flagelle libre dont la présense ou l'absence est pourtant considérée comme le seul grand signé distinctif entre le groupe Congolense et le groupe Vivax. Certes, dans un vrai *Tr. vivax* typique le flagelle libre est long, de même qu'il est complètement absent dans un vrai *Tr. congolense* typique. Mais il n'en est pas de même lorsqu'il s'agit d'un *Tr. uniforme*, d'une part, et d'une longue forme de *Tr. congolense*, d'autre part. Chez le premier, le flagelle libre est parfois bien court, et chez le second on peut parfois observer un vestige de flagelle libre. Dans ces cas la position du blépharoplaste est certes un excellent moyen adjuvant de distinction, ou de détermination.

En somme, morphologiquement, *Tr. simiae* ressemble beaucoup à *Tr. congolense*. Ce qui distingue le premier, ce sont en somme : sa taille un peu plus grande (et encore, certaines formes longues de *Tr. congolense* atteignent 18 μ), la présence parfois d'un moignon de flagelle libre (et encore ! cela s'observe souvent dans les longues formes de *Tr. congolense*) et, enfin, la membrane ondulante très plissée. Mais en ce qui concerne la membrane ondulante, elle est certainement plus développée ou moins développée, ou plus plissée ou moins plissée dans certaines formes de *Tr. congolense*. Les figures de ce dernier trypanosome, données par divers auteurs, sont vraiment frappantes sous ce rapport. Le *Tr. congolense* de Laveran et Mesnil, dont la souche provient du Congo (Léopoldville) a une

membrane ondulante très peu développée. Il en est de même des formes de *Tr. congolense* que nous avons vues nous-même dans le Katanga et à Stanleyville. Il en est de même des formes de *Tr. congolense* reproduites dans les figures du travail de Curson et provenant du Zululand. Par contre, les *Tr. congolense* de Bruce (du Nyassaland) ont une membrane ondulante presqu'aussi développée et plissée que les trypanosomes polymorphes : *Tr. brucei, Tr. gambiense*, etc. . . .

Cela pour la morphologie de *Tr. simiae*, autant que nous puissions en juger d'apres les descriptions et les figures des autres (Bruce et collaborateurs), ne l'ayant personnellement jamais vu. En ce qui concerne ses propriétés biologiques, pour ainsi dire, *Tr. simiae* est extrémement pathogène pour le porc et pour le singe. Il est également transmissible aux moutons, mais pas aux petits animaux de Laboratoire (cobaye, lapin, etc.) ni même au bœuf, ce qui le distingue de *Tr. congolense* et de *Tr. brucei*.

Au point de vue biologique, *Tr. rodhaini* est également très pathogène pour le porc et pour le singe. Il n'est pas transmissible aux petits animaux de Laboratoire. Un seul bœuf d'expérience ne s'est pas infecté, d'après Walravens (ce qui n'est d'ailleurs pas suffisant pour conclure). La transmissibilité au mouton n'a pas été expérimentée. De sorte que sous ce rapport le trypanosome décrit par Walravens ne se distingue pas en somme de *Tr. simiae*.

Mais au point de vue morphologique, *Tr. rodhaini* semble se distinguer des trypanosomes connus, surtout par sa minceur vraiment extraordinaire. Son blépharoplaste latéral le rapproche de *Tr. congolense* (et de *Tr. simaiae*) ; sa longueur ne dépasse pas en somme celle des longues formes de *Tr. congolense* (et de *Tr. simiae*) mais il est beaucoup plus mince et possède en outre un assez long flagelle libre. Ce dernier le rapproche, d'autre part, du groupe Vivax, et tout spécialement de *Tr. uniforme* qui a à peu près la même longueur, mais il est beaucoup plus mince et il a en outre un blépharoplaste latéral.

Répondant à Hornby qui émettait l'avis que *Tr. rodhaini* n'est autre que *Tr. uniforme*, Walravens objecte le fait que son trypanosome ne se déplace pas en flèche, comme ceux du groupe Vivax. Mais cette objection n'en est pas une. *Tr. uniforme* se meut sur place et ne sort pas du champ microscopique. Cela a

été bien spécifié par Bruce et collaborateurs. Nous l'avons également constaté chez les bovidés de Stanleyville où *Tr. uniforme* est assez commun.

Et puisqu'il était—ou même est encore—question d'identifier *Tr. rodhaini* avec *Tr. vivax* et surtout avec *Tr. uniforme*, disons quelques mots de ces deux trypanosomes.

Nous avons jusqu'à présent très peu de renseignements sur la pathogénicité pour le porc des trypanosomes du groupe Vivax, *Tr. vivax* et *Tr. uniforme*. D'après Cazalbou, *Tr. cazalboui* (= *Tr. vivax*) n'est inoculable ni au singe ni au porc. D'après Bouffard, le cercopithèque et le porc sont absolument réfractaires au virus de la souma (*Tr. cazalboui* = *Tr. vivax*). Quant à *Tr. uniforme*, quelques essais de transmission de ce trypanosome furent faits aux ovidés et aux capridés (avec un résultat positif) et au singe, chien, cobaye, rat et souris (avec un résultat négatif) ; mais aucune expérience n'a été faite avec le porc. Comme *Tr. uniforme* ressemble beaucoup à *Tr. vivax*, il est plus que probable que, de même que ce dernier, il n'est pas transmissible au porc. Mais encore faut-il le prouver.

Quoiqu'il en soit, il résulte des quelques expériences faites que *Tr. vivax* n'est transmissible ni au porc ni au singe et que *Tr. uniforme* n'est pas transmissible au singe (ni probablement au porc non plus). Biologiquement donc, *Tr. vivax* et *Tr. uniforme* se distinguent radicalement aussi bien de *Tr. simiae* que de *Tr. rodhaini*, les deux derniers étant pathogènes aussi bien pour le porc que pour le singe.

Au point de vue morphologique, nous n'avons évidemment pas besoin de nous arrêter sur *Tr. vivax*, trypanosome trop connu. *Tr. uniforme* est par contre beaucoup moins connu, et nous croyons par conséquent utile de rappeler bien brièvement sa description donnée par Bruce et collaborateurs et surtout ses signes distinctifs du *Tr. vivax*, sensu stricto.

' Les mouvements à frais de *Tr. uniforme* sont intermédiaires entre ceux de *Tr. vivax* et ceux de *Tr. pecorum* (= *Tr. congolense*), c'est-à-dire *qu'il se déplace dans le champ microscopique.*

Tr. uniforme a une longueur moyenne de 16 microns (12-19), au lieu de la longueur de 23·7 microns (18-31) chez *Tr. vivax*.

Largeur : 1·5-2·5 microns (2-3 microns, chez *Tr. vivax*).

Flagelle libre : 2-6 microns (3 à 6 microns, chez *Tr. vivax*).

Noyau : compact et central (fragmenté et occupant toute la largeur, chez *Tr. vivax*).

Partie post nucléaire : pas aussi nettement rétrécie que dans *Tr. vivax*.

Blépharoplaste : rond et terminal (comme dans *Tr. vivax*).

Membrane ondulante : étroite et peu plissée (comme dans *Tr. vivax*).

En résumé, *Tr. uniforme* ressemble à *Tr. vivax* par son aspect général, mais il s'en distingue par sa taille un peu plus petite.

Qu'on veuille à présent comparer les figures I, II, III et IV, dont la première reproduit *Tr. rodhaini* de Walravens et les trois autres, les trypanosomes trouvés par nous chez divers porcs de Stanleyville et d'Ibembo. On constatera :

1. Que le trypanosome de Walravens est en général plus mince que ceux trouvés par nous, mais que certains des nôtres (et notamment les trypanosomes de la figure 3, B trouvés chez le cochon No. 3 de la Mission) sont aussi minces que *Tr. rodhaini* et ressemblent à tous les points de vue à ce dernier.

2. Que *Tr. rodhaini* a pris chez le singe un aspect un peu différent de celui qu'il avait chez le cochon : est notamment devenu un peu moins mince et un peu moins long et—surtout—son flagelle est devenu beaucoup plus court.

3. Que les trypanosomes trouvés par nous sont un peu différents suivant les troupeaux et même suivant les différents animaux (porcs) du même troupeau.

COCHONS D'IBEMBO (Figure II).

Les trypanosomes du cochon B ressemblent à *Tr. rodhaini*, mais ils sont un peu moins minces et leur flagelle est en général un peu plus court. Mais les trypanosomes du cochon A sont différents et même polymorphes. A côté de petites formes, courtes et assez minces, on voit des trypanosomes longs et assez gros, avec ou sans flagelle libre et surtout avec une membrane ondulante bien développée et bien plissée. Ces trypanosomes ressemblent beaucoup plus à *Tr. simiae* qu'à *Tr. rodhaini*.

A ET B. COCHONS DE LA MISSION (Figure III).

Les trypanosomes du cochon B (cochon No. 3) sont quasi identiques à ceux trouvés par Walravens, tandis que ceux du cochon A

(cochon No. 65) ressemblent plutôt à *Tr. simiae.* Quelques spécimens ressemblent d'ailleurs à *Tr. congolense.*

C. COCHON DE LA TSHOPO (Figure III).

Sang du cœur. Post mortem. Les trypanosomes, tous mono-morphes, peuvent être identifiés avec :

(*a*) *Tr. rodhaini* (quoique plus courts, moins minces et flagelle libre très court).

(*b*) *Tr. congolense* (formes longues avec un moignon de flagelle libre).

(*c*) *Tr. uniforme :* blépharoplaste sub-terminal dans la plupart des spécimens.

COCHON DU TROUPEAU M (Figure IV).

Aussi bien dans ' A ' (cochon noir) que dans ' B ' (cochon rouge) nous avons deux variétés de trypanosomes : avec et sans flagelle libre, d'abord, et—les deux—avec et sans membrane ondulante plissée. Grosso-modo, les trypanosomes de ' B.' ressemblent à *Tr. rodhaini*, tandis que ceux de ' A ' ressemblent plutôt, à quelques exceptions près, à *Tr. simiae.*

On voit donc qu'en nous plaçant uniquement sur le terrain mor-phologique, il est très difficile de déterminer nos trypanosomes des cochons de Stanleyville et d'Ibembo. Quoique cliniquement, ou épidémiologiquement, il s'agissait d'une maladie identique dans tous les cas, l'agent pathogène ne semble pas tout à fait le même dans tous ces cas. A moins que l'agent causal ait un aspect variable, c'est à dire polymorphe, ou qu'il s'agisse, dans certains cas du moins, d'une infection mixte. Dans le sang de nos divers cochons nous avons trouvé toute une gamme de transition entre *Tr. simiae*, décrit par Bruce, et le trypanosome trouvé et décrit par Walravens, en passant par *Tr. congolense* et même par *Tr. uniforme.*

Devant les difficultés rencontrées par nous dans la détermination de nos trypanosomes, nous nous sommes adressés à deux grands spécialistes : à C. M. Wenyon, de Londres, et à P. J. du Toit, de Prétoria.

A Wenyon, nous avons envoyé trois frottis de sang de cochon : un provenant d'Ibembo, un de la Mission et un de la Tshopo. A du Toit, nous avons envoyé deux frottis : un de la Mission et un

d'Ibembo. Les frottis de la Mission provenaient du cochon No. 3 (fig. III, в). Nous ne pouvons dire avec exactitude de quel cochon d'Ibembo provenaient les frottis envoyés. Il est possible que Wenyon et du Toit on reçu des frottis de cochons différents.

Quant au frottis de la Tshopo, envoyé uniquement à Wenyon, il s'agissait de sang de cœur, post mortem, probablement fig. III, c, où les trypanosomes ressemblent tant à *Tr. uniforme*.

Voici un extrait de la réponse de C. M. Wenyon :

' As regards the films ; " cochon I to III ", I have examined the trypanosomes very carefully and am convinced that the infections are due to *Trypanosoma uniforme*. It is highly probable that Walravens' *T. rodhaini* was the same species. In my book, p. 560, I suggested it might be *T. vivax*, but it seems probable that it was *T. uniforme* after what you have found. The trypanosome is the same one in all your films, the appearance differing from pig to pig owing to the fact that dividing forms are more common in some than in others.'

La réponse de du Toit est très peu catégorique :

' The two smears of pig blood containing trypanosomes are indeed very interesting and very difficult to give an opinion on.

' The trypanosomes in your smear No. 2, marked " C I " and obtained from a pig in Ibembo, show, according to our measurements, an average length of 16·5 μ. (13-19 μ). These trypanosomes agree in most characters with *Tr. simiae*.

' The smear from a pig from the mission de Stanleyville, contains trypanosomes which, according to our measurements, vary between 15 and 21μ. with an average length of 17·2μ. In many cases there appears to be a very short free flagellum, about 2μ. in length (0-5μ.). On this latter point, namely the presence of a free flagellum, I am not confident, but should there be a free flagellum we could not call the trypanosome *T. congolense*.

'The trypanosomes in this smear also resemble somewhat *T. simiae*, but it is difficult to decide from one smear whether the trypanosomes only belong to this species.

' Some of the trypanosomes in this smear correspond fairly accurately to *T. rodhaini* of Walravens. It seems to me not improbable that we have in this smear a mixed infection of two or more of the species mentioned above.

Et voici la remarque finale, bien intéressante, de du Toit :

' I must add to this that I am most reluctant to increase the number of names which have been given to species of trypanosomes. In many cases the names can be dropped, but there are great difficulties in regard to some of the trypanosomes, especially those of pigs which, as you remark, are very imperfectly known. I certainly think that a further study of this subject would be well worth while, and would probably throw much light on the confusion which now exists in the literature.'

<p style="text-align:center">*   *   *</p>

Si morphologiquement nous ne sommes pas parvenus à déterminer avec précision les trypanosomes trouvés dans nos divers troupeaux de suidés, les quelques essais de transmission de deux de nos souches nous ont donné des résultats bien bizarres.

Qu'on veuille bien examiner la figure V.

' A ' reproduit les trypanosomes trouvés chez un mouton, le 17ème jour après lui avoir injecté du sang du cochon ' Mission No. 3 ' (figure III, B). Qu'est ce que ce bizarre trypanosome ? C'est un très gros *Tr. congolense* qui ressemble singulièrement à *Trypanosoma Montgomeryi* ! Provient-il vraiment du cochon ? C'est peu probable. Nous rappellerons que ce n'est qu'une seule fois, le 17ème jour après la transmission, que nous avons trouvé ce trypanosome. Le mouton ayant appartenu à un troupeau infecté de *Tr. congolense* (et de *Tr. vivax*), il est plus que probable qu'il s'agissait d'une réapparition fugace d'une ancienne trypanosomiase de ce mouton.

' B ' et ' C ' représentent les trypanosomes trouvés dans le sang d'un mouton, respectivement le 8ème et le 9ème jour après avoir reçu du sang virulent du ' cochon rouge M ' (fig. IVB).

' D ' et ' E ' représentent les trypanosomes trouvés chez un petit cochon, respectivement le 4ème jour et le 9ème jour après avoir reçu du sang virulent du même cochon rouge M. Ici, la réussite de la transmission, aussi bien au cochon qu'au mouton, ne présente donc pas le moindre doute. Et quels trypanosomes avons nous trouvé chez ces deux bêtes de transmission ? Ils n'ont pas l'air de ressembler à *Tr. simiae*, surtout faute de membrane ondulante plissée. Dans ' B ' et surtout dans ' D ', on pourrait hésiter entre *Tr. uniforme* et *Tr. congolense*. Mais dans ' C ' et dans ' E,' il s'agit de *Tr. congolense* (forme longue). Les trypanosomes plus courts, au début, semblent

s'allonger avec la durée de l'infection. La plupart des spécimens n'ont pas de flagelles libres, mais quelques uns en possèdent un, très court d'ailleurs, comme dans la souche initiale chez le cochon rouge.

RÉSUMÉ

Nous ne tirons pas de conclusions de notre étude. Nous ne mettons pas d'étiquette sur les trypanosomes trouvés chez nos cochons. Nous ne créons surtout pas de nouvelles espèces. Nous avons trouvé des trypanosomes ressemblant aussi bien à *Tr. simiae* qu'à *Tr. rodhaini* et occupant morphologiquement une place intermédiaire entre ces deux ' espèces.' Certains spécimens ressemblaient à *Tr. uniforme*. Par les quelques rares essais de transmission réussis nous avons obtenu plutôt des *Tr. congolense.*

Toutes ces constatations sont certes bien bizarres. Et la seule conclusion qu'on peut tirer de tout cela, c'est que les trypanosomiases des suidés sont encore à classer, de même qu'il faut reviser, en confirmant ou en infirmant, la spécificité de *Tr. simiae*, Bruce, et de *Tr. rodhaini*, Walravens.

Nous soumettons notre matériel et nos considérations à la discussion.

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PLANCHE VI

LISTE DES FIGURES

Figure I. *TRYPANOSOMA RODHAINI* WALRAVENS.

  A. Sang de cochon.

  B. Sang de singe.

Double figure faite d'après les frottis envoyés par le Docteur Walravens.

Figure II. TRYPANOSOMES DE COCHONS D'IBEMBO

  A. Trypanosomes trouvés dans un frottis envoyé par le Docteur Henneaux.

  B. Idem. Un autre frottis provenant d'un autre cochon.

Figure III.

  A. Cochon No. 65 de la mission St. Gabriel.

  B. Cochon No. 3 de la mission St. Gabriel.

  C. Cochon No. 3 de la Tshopo. Sang du cœur post mortem.
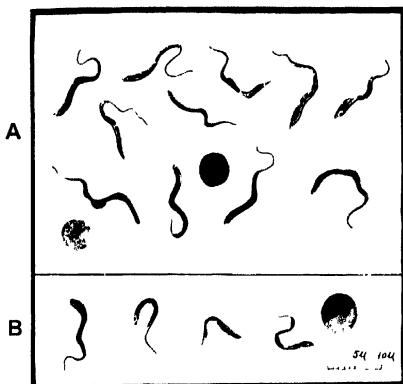
FIG. I.
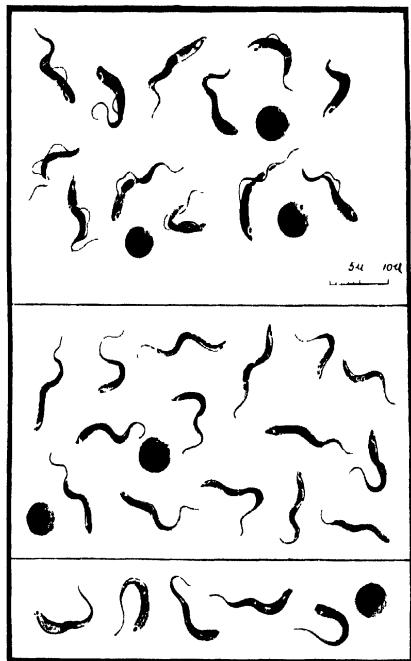
FIG. II.
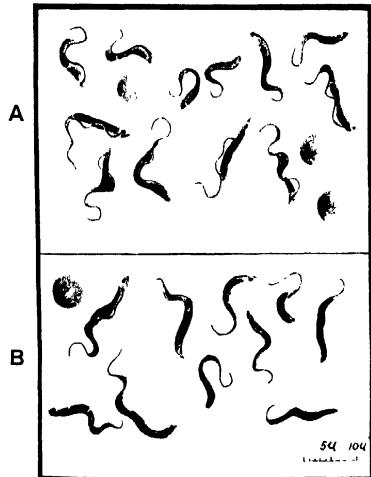
FIG. III.

PLANCHE VII

LISTE DES FIGURES

Figure IV.

    A.   Cochon noir de la ferme M.

    B.   Cochon rouge de la ferme M.

Figure V.   ESSAIS DE TRANSMISSION.

    A.   Sang de mouton, le 17ème jour après avoir reçu du sang virulent du cochon ' No. 3 Mission ' (fig. III B).

    B. et C. Sang de mouton, 8ème et 9ème jour après avoir reçu du sang virulent du ' cochon rouge M ' (fig. IV B).

    D. et E. Sang de cochon, 4ème et 9ème jour après avoir reçu du sang virulent du même ' cochon rouge M.'
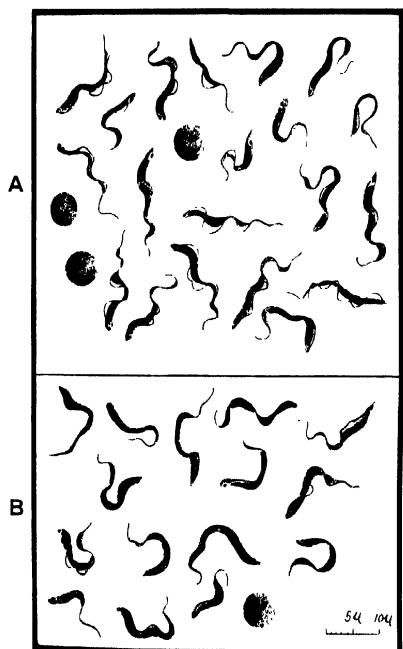
FIG. IV.
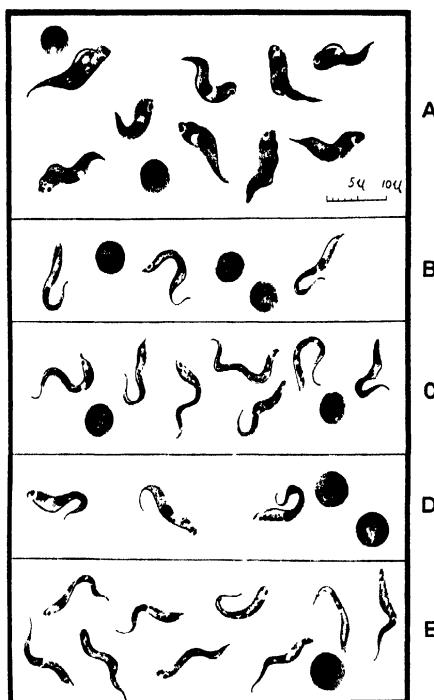
FIG. V.

ON A NEW *DACTYLOSOMA* OCCURRING IN FISH OF VICTORIA NYANZA

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INTRODUCTION

While working at the Human Trypanosomiasis Research Institute, Entebbe, Uganda (1927-1929), I had the opportunity of examining a number of fish from Lake Victoria, belonging to the genus *Haplochromis*. These small fish, only a few inches long, are known locally as 'Enkeje.' They are caught in large numbers by the Baganda fishermen, dried in the sun and used as an article of diet. The species examined by me for parasites were *Haplochromis humilior, H. nubilus, H. cinereus, H. serranus* and *H.* sp.* Members of all these species, except the first named, were found to be infected with a *Dactylosoma*.

As is known, the genus *Dactylosoma* is represented by intracellular blood-parasites belonging to the order Coccidiida ; its precise systematic position has not been definitely established, but it appears to be related to both the Haemosporidiidea and the Piroplasmidea. Hitherto these parasites have been recorded only for amphibia (frogs and newts). Wenyon (1926) believed that the organism found by Awerinzew (1914) in *Chamaeleon fischeri* and named *Lankesterella amania* was allied to *Dactylosoma* and he accordingly changed its name to *D. amaniae*. Dr. Wenyon informs me, however, that he was unable to consult Awerinzew's original publication—which, as far as I am aware, is unobtainable in this country—and his conclusions were, therefore, based on an abstract of the paper. Through the courtesy of Dr. H. Epstein, of Moscow University, I had the opportunity of consulting Awerinzew's work in Russian and was

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* I wish to acknowledge my indebtedness to Mr. J. R. Norman, of the British Museum (Natural History) for the identification of these fish.

able to confirm Wenyon's contention that the chameleon parasite does not belong to the genus *Lankesterella*. On the other hand there do not appear to be sufficient grounds for referring it to *Dactylosoma*.

The journal in which Awerinzew's paper was published being inaccessible to English readers, the following free translation of his description of the chameleon parasite may be of interest.

'I was able to follow the vegetative multiplication of the haemogregarine described with sufficient completeness in blood-films of *Chamaeleon fischeri*. One usually finds in the erythrocyte only one haemogregarine representing a very small globule with a sharply stained central body. No membrane was found around the parasite. The central sharply-staining body I regard as a nucleus and the thin external layer surrounding this body—as the protoplasm. The haemogregarine gradually grows and increases in volume, but no sharp differentiation of the nuclear and protoplasmatic parts can be observed in it. There is no accumulation of pigment or any other alterations in the parasite, nor does it produce any visible destructive action upon the erythrocyte. When the haemogregarine reaches a fairly large size division of its nucleus leading to the breaking up of the entire organism into parts can be observed. The nucleus divides at once into six nuclei. The final stages of this division resemble somewhat similar stages in the division of the nucleus of *Calcituba polymorpha* [Foraminifera], described by Schaudinn. From the central body to the periphery are given off filaments on the outer ends of which the chromatin particles accumulate. Finally, the entire central body disappears, instead of which there appear six separate bodies on the periphery of the parasite, after which the entire parasite breaks up into six individuals. . . . The parasite described by me should probably be referred to *Lankesterella*. I propose to name it *Lankesterella amania*.'

Awerinzew does not give any measurements of his parasite. From his figures it can be estimated that the diameter of the undivided' parasite is about 1/25 to 1/17 of the long axis of the host-cell, while that of the schizont is 1/8. From blood-films of *Chamaeleon gracilis* in my collection I found that the long axis of its erythrocyte measures on the average about 20μ. Assuming the red-cells of *C. fisheri* to be equal in size to those of *C. gracilis*, the measurements of Awerinzew's parasite would be about 0·8μ to 1·2μ for the undivided stages, and 2·5μ for the schizont. It is thus considerably smaller than any of the known species of *Dactylosoma*, including the one described here. It also differs from members of this genus in other respects. In none of them is the trophozoite spherical with a central nucleus, nor is division of the type described by Awerinzew; moreover, the nucleus of *Dactylosoma* is always sharply differentiated from the cytoplasm. It is impossible from Awerinzew's description and figures to assign ' *L. amania* ' to any known group of haemoprotozoa.

Before any conclusion can be arrived at it will have to be re-investigated. At present all that can be done is to include this parasite among the already numerous intracellular organisms, *incertae sedis*, of unknown—animal or vegetable—affinities.

The present paper records for the first time a *Dactylosoma* in fish and thus extends the distribution of this genus to a second class of cold-blooded animals.

Owing to the similarity of certain stages of *Dactylosoma* to other haemococcidian parasites, some authors have included them in various other genera, such as *Haemogregarina*, *Lankesterella*, *Laverania*, etc. The question has been thoroughly discussed by Nöller (1913), who provided a list of synonyms of the frog-parasite, *D. ranarum*, the only form which has been thoroughly investigated.

DESCRIPTION

The total number of fish examined by me was fourteen, of which five—belonging to the four species named above—were infected.

The parasite was studied in blood-films and organ-smears fixed with methyl alcohol and stained with Giemsa's mixture.

All the stages of asexual development occur in the peripheral blood. Erythrocytes exclusively were found to be infected, and there was no evidence of the parasites affecting the host-cells in any way. According to Nöller (1913), *D. ranarum* causes dehaemoglobinization of the red cells, while Mathis and Leger (1911) found them unaltered.

The various stages of the parasite observed can easily be arranged in the order of their development in the vertebrate host. The merozoites that had apparently recently penetrated into the host-cells measure 2.8μ by 0.9μ (fig. A, I, 2, 3).

They are slightly larger than merozoites which have evidently just been formed and have not yet left the cell in which schizogony occurred (fig. B, 24).

These merozoites or trophozoites, as they may be termed at this stage, have the shape of short rods, usually slightly curved. One end of the body is somewhat narrower than the other, the nucleus being situated at the broader end. At a later stage the trophozoites
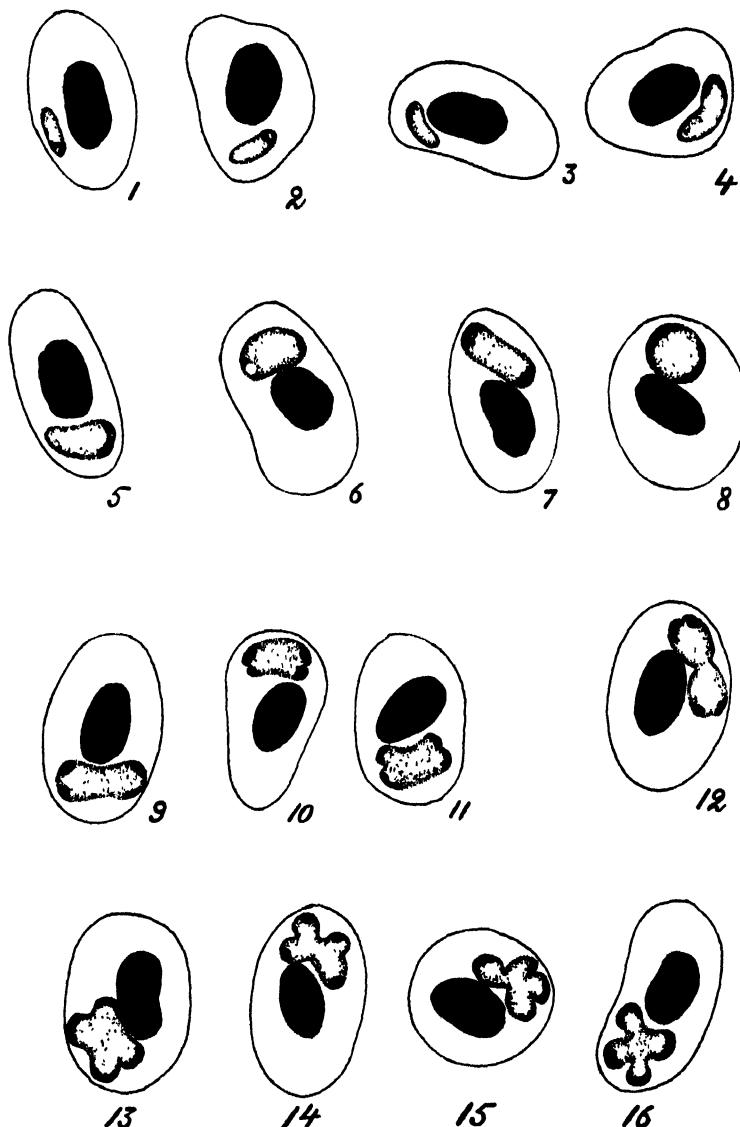
C. A. Hoare, del.

FIG. A. *Dactylosoma mariae* sp.n. 1, 2, 3.—Merozoites; 4.—Trophozoite; 5, 6.—Early schizonts; 7, 8.—Beginning of schizogony : binucleate stage; 9, 10, 11.—Schizogony : tetranucleate stage; 12-16.—Schizogony : differentiation of merozoites. All × 2000.

increase in size, measuring from $3\cdot8\mu$ to $5\cdot7\mu$ in length, and from $1\cdot9\mu$ to $5\cdot0\mu$ in breadth. These forms are probably early schizonts (fig. A, 5, 6).

Schizogony is initiated by the division of the nucleus into two parts which become disposed at opposite poles (fig. A, 7, 8). Each nucleus then divides into two, the two pairs still retaining their polar arrangement (fig. A, 9-13). At this stage the body gradually becomes constricted in the middle, while a cleft appears at each pole separating the two nuclei. This process represents the differentiation of the merozoites corresponding to the four nuclei. As the division progresses the schizont loses its bipolar symmetry, the daughter-elements becoming more or less radially arranged (fig. A, 13-16). When the clefts between them reach the centre, the schizont assumes a more or less regular rosette shape, similar to that of some piroplasms, the daughter-forms being piriform with the nucleus at the broad distal end (fig. B, 18-20). Finally the connection between the merozoites is severed and they come to lie free in the cell (fig. B, 21, 22). There is no residue after division, the entire substance of the schizont being used up in the formation of the merozoites. The newly-formed merozoites have the original rosette arrangement, but later become scattered within the host-cell, while the body becomes elongated as in the young trophozoites (fig. B, 24).

The regular schizogony described is predominant and apparently represents the normal type, but occasionally deviations from this are encountered. Thus, a schizont may be fan-shaped with the nuclei disposed along the border of the broader side (fig. B, 23); the formation of the merozoites may not be synchronised, some developing more rapidly than others, and the process being more like true budding (fig. B, 17, 20, 24). The early stages of schizogony —before rosette formation—measure from $4\cdot7\mu$ to $5\cdot7\mu$ in length, and from $1\cdot9\mu$ to $3\cdot8\mu$ in breadth.

Schizogony in this parasite is characterised by the formation of *four* radially arranged merozoites. Neither more nor fewer have been observed. It differs from the *Dactylosoma* of frogs in that the latter has two types of schizogony. In one, numerous merozoites are produced and are arranged unilaterally, the fully-developed schizont having the appearance of a hand, the fingers of which are represented by the merozoites (hence the name of the genus). In the second
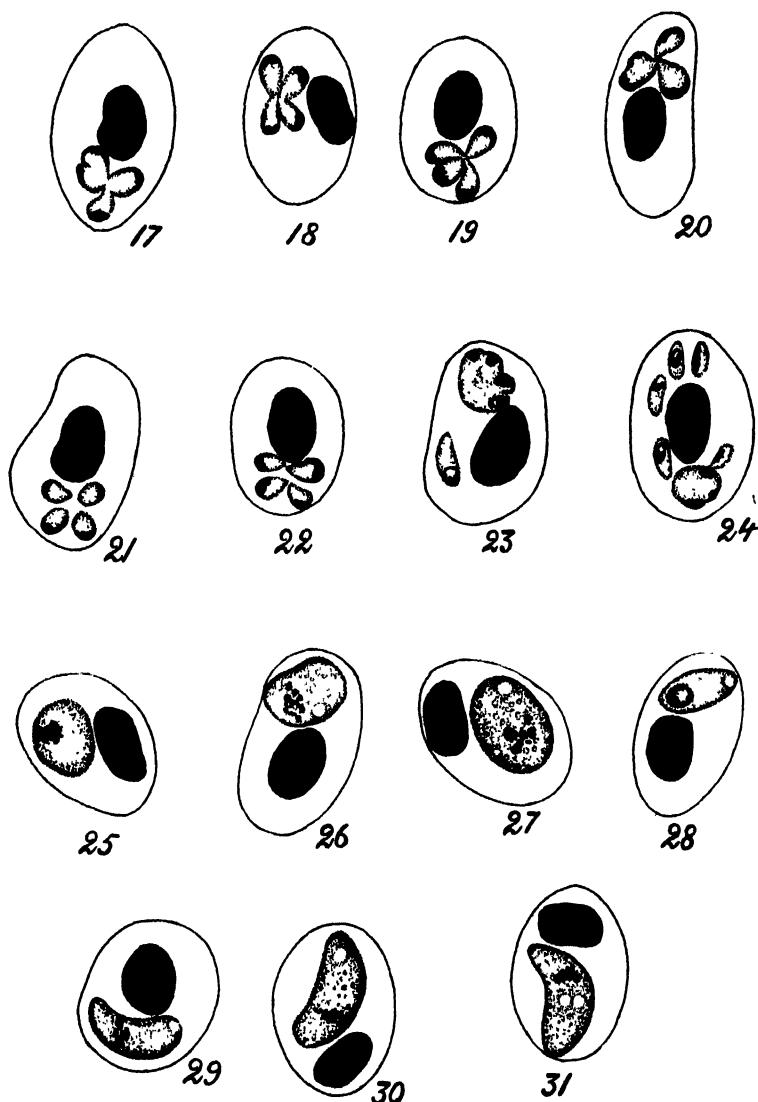
C. A: Hoare, del.

Fig. B. *Dactylosoma mariae* sp.n. 17-20.—Final stages of schizogony : merozoites in rosette formation ; 21-22.—Separation of merozoites ; 23.—Double infection of host-cell : (a) schizont with unilateral arrangement of nuclei,' and (b) a merozoite ; 24.—Double infection of host-cell : (a) schizont showing unusual form of division : merozoite budding off. (b) Four sister-merozoites completely separated ; 25.—Young female gametocyte ; 26-27.—Mature female gametocytes ; 28.—Young male gametocyte ; 29, 30, 31.—Mature male gametocyte. All × 2000.

type a few merozoites only (four to six) are produced, their arrangement being more or less radial (Nöller, 1913: *Dactylosoma ranarum ;* Dutton, Todd and Tobey, 1907: ' *Drepanidium* ' (= *Dactylosoma*) from *Rana galamensis*) The second type of division corresponds to some extent with the type described here, for the parasite of fish, except that in the latter the number of merozoites is constantly four. A similar type of schizogony was recently described by Nigrelli (1929) in *Dactylosoma jahni* from a newt, *Triturus viridescens*. In this organism the number of merozoites produced is also four, arranged in the form of a rosette. *D. jahni*, however, differs from the parasite described here in that it has also other types of schizogony—one in which the merozoites are formed by successive budding, and another in which a residual body is left after schizogony.

The stages described represent the asexual cycle of development of the *Dactylosoma* of the fish. Apart from these there occur forms which have been interpreted as gametocytes in other members of the genus. These are of two types. In one the body is club-shaped and curved in the form of a crescent (fig. B, 29, 30, 31). The nucleus lies near the middle of the body and there are a few chromatin staining granules (volutin ?) scattered in the cytoplasm. These forms measure from $6·6\mu$ to $8·5\mu$ in length, and from $1·9\mu$ to $3·5\mu$ in breadth. In the second type the body is rounded, the nucleus is also near the middle of the body, and the dark granules are apparently absent. The cytoplasm of this type is more coarsely granular than in the preceding form (fig. B, 26, 27). The rounded forms measure from $5·7\mu$ to $6·6\mu$ in length, and from $3·8\mu$ to $5·7\mu$ in breadth. In both forms the cytoplasm stains more deeply in the asexual stages and contains various minute granular inclusions. Another characteristic feature common to the two forms described is the presence of one or two small vacuoles, though these are occasionally seen in other stages (fig. A, 6). According to Nöller (1913) these vacuoles appear in fresh preparations of *D. ranarum* as refractile globules and probably represent oil-inclusions. The crescent-shaped form resembles the gametocytes of various haemosporidia, and it is probable—as interpreted by other authors—that the two forms are male and female gametocytes, the crescents, according to Nöller, being the male gametocytes and the rounded forms the female ones. The earlier stages of gametocytes

differ from the fully developed ones only in smaller dimensions (fig. B, 25, 28).

Nothing is known regarding the intermediate hosts or method of transmission of any *Dactylosoma*.

Apart from the characters noted already (type of schizogony and number of merozoites), the *Dactylosoma* described here differs from other known members of the genus in the dimensions of the various stages. The characters of this parasite and its occurrence in a new class of host are sufficient to distinguish it as a new species for which the name *Dactylosoma mariae* sp.n., is proposed.

DIAGNOSIS AND SUMMARY

Systematic position. *Dactylosoma mariae* sp.n. (Coccidiida, near Piroplasmidea).

Habitat. Parasitic in erythrocytes of *Haplochromis nubilus*, *H. cinereus*, *H. serranus* and *H.* sp. (Pisces Teleostei).

Locality Victoria Nyanza, near Entebbe, Uganda.

Description. Asexual development (schizogony) and formation of gametocytes in the peripheral circulation of the host. Merozoites, $2\cdot8\mu$ by $0\cdot9\mu$; trophozoites, $3\cdot8\text{-}5\cdot7\mu$ by $1\cdot9\text{-}5\cdot0\mu$; schizogony resulting in production of four merozoites arranged in the form of a rosette, without residual body. Gametocytes of two types (δ and $\female$) crescent-shaped ($6\cdot6\text{-}8\cdot5\mu$ by $1\cdot9\text{-}3\cdot5\mu$) and rounded ($5\cdot7\text{-}6\cdot6\mu$ by $3\cdot8\text{-}5\cdot7\mu$). Intermediate host and sporogony unknown.

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STUDIES ON THE ACTION OF YELLOW FEVER VIRUS IN MICE

BY

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The study of the etiology of a disease is greatly facilitated by the discovery of an experimental animal susceptible to the virus of that disease. The finding by the West African Yellow Fever Commission of the Rockefeller Foundation (1928) that the Indian monkey *Macacus rhesus* is susceptible to the virus of yellow fever has been productive of a great deal of research with fruitful results. Rhesus monkeys, however, are rather expensive and at times the supply is limited. The finding of a common laboratory animal susceptible to the virus of yellow fever would be a great advantage.

It is generally conceded by all workers that the common laboratory animals,—rabbits, guinea-pigs, rats and mice, are not susceptible to the virus of yellow fever when injected by the usual routes.

The French workers Lasnet (1929) and Laigret noted in yellow fever patients neurological symptoms. Laigret suggested that the central nervous system of yellow fever patients should be tested for the presence of virus.

It was decided to test the common laboratory animals by intra-cerebral injection. Familiarity with the work of Andervont (1929) with herpes virus and mice, and the simplicity of his technique suggested mice as the first choice.

PRELIMINARY EXPERIMENTS

In the preliminary experiments material from three different rhesus monkeys dying of yellow fever was employed. In every case mice were injected intracerebrally and in two cases the same material was injected intraperitoneally. The results of these experiments

TABLE I.

Mice injected intracerebrally with Yellow Fever Virus from Rhesus Monkeys.

Preliminary Experiments.

Number of rhesus	Material injected	Number of mice	Result	Remarks	Microscopic pathology
I	Brain.	1	Killed, 8th day.	No lesions.	No tissues saved.
		2	Ill, 10th day.	Ether. Brain proved infective to four mice which all died on 8th and 9th days.	No tissues saved.
		3	Ill, 19th day.	Ether. No lesion.	No tissues saved.
		4	Found dead, 21st day.	...	No tissues saved.
II	Citrated blood.	1	Ill, 11th day.	Ether. Brain proved infective to two mice, dying on 8th and 9th days.	No tissues saved.
	Brain.	1	Ill, 12th day.	Ether. Brain proved infective to two mice, both dying on 8th day.	Encephalitis.
		2	Dying, 23rd day.	...	No tissues saved.
		3	Remained well.	. .	...
		4	Remained well.	...	...
		5	Remained well.	...	...
III	Brain.	1	Died, 1st day.	...	No tissues saved.
		2	Ill, 5th day.	Ether.	Negative.
		3	Ill, 6th day.	Ether. Passed to five mice which all remained well.	Negative.
		4	Found dead, 18th day.	...	No tissues saved.
		5	Ill, 21st day.	Ether.	Encephalitis.
		6	Ill, 22nd day.	Ether.	Encephalitis.
		7	Found dead, 25th day.	...	No tissues saved.
		8	Found dead, 25th day.	...	No tissues saved.
		9	Ill, 25th day.	Ether.	No tissues saved.
		10	Ill, 30th day.	Ether.	No tissues saved.
		11	Remained well.	...	...
		12	Remained well.	...	...

are summarized in Table I. The material used consisted of brain from one monkey, citrated blood and brain from another, and brain from a third All the mice which received an intraperitoneal injection remained well and are not included in the table. Of the mice which received an intracerebral infection of citrated blood, only one survived the injection, as citrate appears to be very toxic when injected by this route. Examination of the table shows that quite a number of the mice became ill. Furthermore, the virus could be propagated in other mice by brain to brain passage. In three out of four cases in which this was tried the mice became infected and died after an appreciably shorter course of the disease. Two of these strains were stopped after the second passage and the third died out for some unknown reason in the fourth generation.

Pathological study was made of brains from five mice, injected with virus from the second and third monkeys. Three of these brains showed an acute encephalitis and two were normal. It is significant that one of these which was negative pathologically was also negative on passage to other mice. No brains were preserved from mice injected with material from the first monkey. The results of these preliminary experiments were sufficiently encouraging to warrant a more exhaustive study. The remainder of this article is concerned with the work done with a fourth strain established in mice.

ESTABLISHMENT OF THE VIRUS OF YELLOW FEVER IN MICE

I am indebted to Dr. A. W. Sellards for the strain of yellow fever virus used in these experiments. This virus originated in Senegal and is known as the ' French Strain ' in medical literature.

Six mice were injected intracerebrally with the serum of a monkey which was dying from yellow fever. One of these mice was found dead the following day. Of the five remaining mice, one was definitely ill on the eighth day, one was found dead on the tenth day, one was ill on the fourteenth day and two remained well. The brain of the mouse which was ill on the fourteenth day proved non-infectious for other mice. The mouse which appeared ill on the eighth day was killed by means of ether and the brain used for

passage of the virus to other mice and for pathological study. Microscopical examination showed an encephalitis and the presence of eosinophilic intranuclear bodies. Five mice were injected intra-cerebrally with a portion of this brain and all were dead or dying and were killed from the seventh to the ninth day. The virus has since been kept continuously in mice for more than seventy-five passages, over a period of fourteen months.

In this paper, whenever the term ' virus ' is used, the strain established in mice is meant, unless definitely indicated otherwise.

TECHNIQUE

The methods employed were simplified as much as possible. For the preparation of the virus for passage the brain is removed from an infected mouse by reflecting the skin over the head, then searing the surface of the cranium and opening the skull by means of sterile scissors. The brain is removed and cut up into small pieces, saline is added, usually about 5 c.c. per brain, and a suspension is made by forcibly drawing the brain susbtance in and out of a syringe through a rather coarse needle. This suspension is allowed to stand for several minutes in order that the larger particles may settle. The supernatant fluid is used for injection purposes. Two drops of this is the usual dose and 0·5 c.c. is the maximum that can be injected intracerebrally with safety. The skin of the head is washed with alcohol and the injections are made through the skin and bone at the posterior end of the skull, a little to one side of the mid-line. After an intracranial injection · some mice show great excitement, others lie very quiet as if dying. These conditions may last for several hours, but the following day the mice appear to be perfectly well and remain so until they become ill as a result of the infection. Crude as this method is, it is remarkable how few secondary infections occur.

COURSE OF INFECTION

After the shock due to the injection has passed off the mice appear perfectly normal. The first sign of illness is generally a roughening of the fur and a loss of activity. The next day the mouse is usually very ill remaining in one spot and in many cases

showing paralysis of the hind legs. This condition increases until death occurs the same day or the next. In the early passages, the time of onset of illness until the death of the animal was usually two or three days, but sometimes the mouse would linger on in a moribund condition for two or three days longer. In the later passages the mice usually appeared ill on one day and died later the same day or early on the next. It would often occur that all the passage mice were perfectly well in the morning and all dead or dying in the evening of the same day.

At autopsy there are no constant macroscopic changes on which the diagnosis of infection with the yellow fever virus can be based. The only sure means of diagnosis is the examination of sections of brain, which invariably show an encephalitis and specific nuclear changes. These changes are described in more detail later in this paper.

PASSAGE

The strain upon which this study is based has been maintained continuously in mice since November 8, 1928, and is now at the time of writing (January, 1930) in the seventy-fifth passage. In the early passages, the virulence increased rather rapidly up to the fifth passage when all the mice became ill on the sixth or seventh day and died on the seventh or eighth day after injection. After that the virulence increased more gradually. At the twentieth passage the incubation period was on the average one day shorter and most of the mice were dying on the sixth or seventh day. Later still, at the thirtieth passage, the incubation period was one day less and most of the mice were in a dying condition on the sixth day. Transfer was made when the mice were obviously ill or dying. At the time of writing the passage mice are usually all dead or dying on the fifth day. The average interval between the successive transfers in the first twenty-five passages was 6·3 days, in the next twenty-five passages 5·7 days, and the third twenty-five passages 5·1 days.

In the earlier passages, occasionally a mouse injected intracerebrally with passage virus escaped infection. These all proved to be susceptible when given a second injection. It is felt that some of these failures were due to poor technique. Later, when the technique was well established, no failures occurred. In view of the

fact, however, that in the earlier experiments an occasional mouse failed to become infected no mouse was considered to have been immunized unless it survived two later intracerebral injections of passage virus. Up to date, no normal mouse has been found to be immune to the established virus and many hundreds have been used. After repeated passages the virus became so virulent that if the hypodermic needle was merely dipped into the infective brain suspension and then thrust into the brain of the mouse, infection occurred.•

INTRAOCULAR INJECTION

On account of the small size of the eyes of mice, it is possible to inject only a very small quantity into the cavities of the eye. Furthermore it was found impossible to gauge accurately whether the injection was made into the anterior or posterior chambers. Mice injected into the eye nearly always died two or three days after the control mice injected with the same material into the brain. The few that survived did not prove to be immune to a later intracerebral injection of the virus. Brains of mice infected by the intraocular route present the usual pathological picture. Scarification of the cornea and rubbing in of the virus in the few cases in which this procedure was adopted did not result in infection or the development of immunity.

INTRASPINAL INJECTION OF THE VIRUS

In addition to the intracerebral route mice can be infected by injecting the virus intraspinally. This method has the advantage that larger quantities can be injected, but has the disadvantage that the technique is more difficult and one is never sure that the point of the needle is in the spinal canal. Ten mice were injected intraspinally ; six· died and four remained well. These four were later shown to have been immunized when tested by an intracerebral injection of passage virus. It is possible that in these four mice the injection was not made into the spinal canal. As will be shown later, an injection of the virus into the muscles of the back leads to the development of immunity. The course of the disease in the mice which become infected after an intraspinal injection was

longer and the time of death later than in the control mice injected into the brain with the same virus. The pathological changes in the brains of mice infected by an intraspinal injection are the same as those found after an intracerebral injection.

INJECTION OF ADULT MICE INTRAPERITONEALLY WITH VIRUS

Adult mice injected intraperitoneally with massive doses of virus as a rule remain well. Of a large number of mice injected in this manner, in many experiments, only two died. The brains of both of these showed the typical encephalitis. It may be significant that both these brains also showed the presence of a protozoon-like organism, which is probably an *Encephalitozoon*. Intraperitoneal injection of virus leads in about two-thirds of the injected animals to the development of an immunity to an intracerebral injection of passage virus. The development of immunity is not dependent on the amount of virus injected. In one experiment three mice were given an intraperitoneal injection of 0·4 c.c. of virus and four mice received 0·05 c.c. of the same virus. When tested twenty-four days later, only one of the three mice which received the 0·4 c.c. was immune, whereas three of the four mice which received the smaller injection were immune.

The action of an immune serum can be demonstrated indirectly by intraperitoneal injection. Of six mice which received 0·4 c.c. of a mixture containing equal parts of virus and immune human serum, none developed an immunity when tested eleven days (three mice) and eighteen days (three mice) afterwards. Six mice received a similar mixture intraperitoneally except that normal human serum was used. One of these died on the seventh day as a result of infection with the virus. Of the five survivors, the immunity of three was tested on the eighteenth day and two on the forty-fourth day. Of those tested on the eighteenth day, one died on the twenty-second day and two remained well. Both of those tested on the forty-fourth day remained well Thus, none of the mice which received the immune serum-virus mixture developed an immunity, whereas of the six mice which received the normal serum-virus mixture, one became infected and four developed an immunity.

OTHER ROUTES OF INJECTION

All mice injected on various occasions into the skin of the pad of the hind foot remained well. When tested by an intracerebral injection of passage virus fourteen to twenty-four days after the intradermal injection, approximately one-half were found to have developed an immunity. Scarification of the skin of the abdomen and rubbing in the virus produced an immunity in one out of six mice.

Intramuscular injection (muscles of the back, three mice ; masseter muscle, two mice) of the virus into five mice did not produce any infection and death, but all five mice showed an immunity when tested twenty days afterwards.

Intratesticular injection of the virus into five mice produced immunity in three mice.

LOCALISATION OF THE VIRUS IN MICE DYING AFTER AN INTRACEREBRAL INJECTION

Various experiments were undertaken to determine if the virus spread throughout the infected mouse after an intracerebral injection. Tissues were removed from infected mice, ground up with saline in a mortar, and injected intracerebrally into normal mice. In addition to blood, organs thus tested were spleen, liver, kidney, testis and adrenal. In every case in which the infectivity of an organ was tested, some portion of the nervous system of the same mouse, usually the brain, was also tested. Summarizing these experiments it may be said that the blood, liver, spleen, kidney and testis of a mouse dying after an intracerebral injection contain either very little or no virus. Very few of the mice injected with suspensions of these organs became ill and died. Several mice which appeared ill were etherized and their brains proved to be negative upon histological examination. By far the greater majority remained well and were later given an immunity test by the injection of virulent passage virus. All died within the same range of time as the normal controls.

In marked contrast to the organs above mentioned was the adrenal gland. This organ proved to be highly infectious. Thus in one experiment five out of six mice became ill and died, or were

etherized for pathological study after an intracerebral injection of rather a dilute suspension made from one adrenal gland. Four of these brains were examined microscopically and all showed the typical encephalitis. Mice injected with a heavy suspension of testis or kidney of the same animal all remained well.

The spinal cord and sciatic nerve of a mouse dying after an intracerebral injection of virus always proved to be highly infectious. It will thus be seen that at death the whole nervous system and the adrenal gland contain the virus. It will be recalled here that the medulla of the adrenal is developmentally associated with the sympathetic ganglia.

The death of a few mice after an intracerebral injection of abdominal organs could be explained by the presence of the virus in the nerve tissue in those organs. But as mentioned above, sections of the brains of such mice were negative pathologically. It is also possible that the haemorrhages into the stomach may be referred to lesions of the nerves supplying that organ.

CENTRIFUGAL PASSAGE OF THE VIRUS IN THE NERVOUS SYSTEM

That the virus probably travels along the nervous routes can be shown by testing the infectivity of various parts of the nervous system at different intervals of time after an intracerebral injection. Thus the spinal cord is already highly infectious three days after an intracerebral injection of virus, whereas the sciatic nerve and adrenal gland at this time are non-infectious. Later, at the fifth and sixth days, when the mouse is dying of the infection, the virus is present in large quantities all through the nervous system, sciatic nerve and adrenal included.

INFECTION OF YOUNG MICE

Various attempts were made to infect mice by intraperitoneal injection of the virus. This procedure in adult mice produced infection and death only in exceptional cases. Young mice, however, from birth up to about two weeks of age, were found to be susceptible. The time of death in young mice after an intraperitoneal injection was slightly later, from one to five days, than in

the control adult mice injected intracerebrally with the same virus. In all thirty-eight mice, varying in age from three days to two weeks, were given an intraperitoneal injection of virus from nine different mice. Of these, ten were killed during the incubation period, twenty-four became infected and died, or were killed *in extremis*, and four remained well. Three of the four which remained well were later shown to have developed an immunity. Mice more than two weeks old reacted to the virus like adult mice and very seldom succumbed to an intraperitoneal injection. In the earlier experiments occasionally a mouse did not become infected ; later, however (after the twenty-sixth mouse brain passage), no mouse less than two weeks old failed to become infected and die. Also, in the earlier experiments, the mice often lived any time up to thirteen days after injection, whereas in the later experiments, they usually died on the sixth or seventh day. There is thus noticeable in these intraperitoneal injection experiments the same increase of virulence as is shown by the brain to brain passage.

The signs of infection in young mice after an intraperitoneal injection are the same as those observed in adult mice after an intracerebral injection. There is the same roughening of the fur, the same inactivity and the same paralytic symptoms. Haemorrhage into the stomach in a few mice is the only macroscopic lesion present. The liver and other organs appear normal and, as mentioned in another place, contain very little, if any, virus. Microscopically, the brain shows the usual encephalitis and nuclear changes.

DISTRIBUTION OF THE VIRUS IN YOUNG MICE DYING AFTER AN INTRAPERITONEAL INJECTION

The infectivity of the blood and various abdominal organs of young mice dying after an intraperitoneal injection of virus, was tested by the injection of suspensions of these tissues into the brains of normal mice. All the experiments undertaken showed that the distribution of the virus in these mice was the same as in adult mice dying after an intracerebral injection ; namely, that the blood and the abdominal organs, with the exception of the adrenal which proved highly infective, were relatively free, whereas the nervous system, i.e. brain, spinal cord, and sciatic nerve contained

the virus in large amounts. Of a large number of mice injected with the abdominal organs (excluding the adrenal) only one became ill after a prolonged incubation period. The rest remained well and were later given an immunity test with passage virus to which all succumbed. The one mouse which became ill had received a mixture of kidney and spleen. The brain of this mouse showed the typical encephalitis.

A series of experiments were undertaken to determine the path of infection in young mice after an intraperitoneal injection of virus. For this purpose the infectivity of the various tissues was tested at intervals after an intraperitoneal injection. The results of several experiments of this nature showed that twenty-four hours after an intraperitoneal injection the blood is not infective ; liver, spleen and kidney (these organs were usually ground up together) contained the virus, though not to any great extent. Thus of twelve mice which were injected with a mixture of liver, spleen and kidney, five became ill, three of these died, and two were etherized and the brains preserved for study. Both these brains showed the typical encephalitis. It is not known whether the virus had actually penetrated the organs or whether the positive results were due to the presence of the virus on the peritoneal surface of these organs.

The infectivity of the brain twenty-four hours after an intraperitoneal injection was tested on three different occasions. In one experiment it proved non-infective. In the second it was shown to contain only a small quantity of virus. In this experiment four mice were used and all remained well Two, however, were shown later to have developed an immunity. In the third experiment, two out of six mice became infected. Forty-eight hours after an intraperitoneal injection the localization of the virus is very definitely confined to the nervous system. Liver, spleen and kidney are entirely free of virus. Of twelve mice injected with brain, eleven became ill and died or were killed for pathological study, and one remained well. Four of those which became infected had a rather long incubation period (eight to sixteen days). The results of the infectivity tests seventy-two hours after an intraperitoneal injection were the same as those of the forty-eight hour series, except that the brain contained the virus in larger amounts as manifested by the death of all the mice injected from the fifth to the seventh day.

The infectivity of the sciatic nerves of young mice injected intraperitoneally was tested at two different times,—once on the third day after injection, the second time on the sixth day when the injected mouse was dying as a result of the infection. All of six mice injected with the sciatic nerve removed three days after injection remained well. Four weeks afterwards these mice were given an immunity test ; five died in the same time as the controls and one remained well, demonstrating that the sciatic nerve at this time contained very little virus. Of the four mice injected with a suspension of the sciatic nerve removed six days after an intraperitoneal injection, three became ill and were killed. The brains of all three showed the typical pathology. The fourth mouse remained well and was given an immunity test, but died immediately after the injection. This experiment shows clearly that the sciatic nerve on the sixth day after an intraperitoneal injection of virus contains the virus in a fairly high concentration. The difference of infectivity of the sciatic nerve on the third day and the sixth day, and the absence of the virus in the blood at any time, is good evidence 'n favour of the theory of the propagation of the virus along the nerve

PRESERVATION OF THE MOUSE VIRUS BY FREEZING

Brains removed from mice dying as a result of infection with yellow fever virus were stored in the cold room at $-8°$ C. The infectivity was tested on several occasions up to 160 days. The results may be summarized as follows. Infective mouse brain stored at a temperature of $-8°$ C. retains its virulence for at least 160 days. There is, however, a gradual loss of virulence as manifested by a prolonged incubation period in several mice and the failure of some mice to become infected. Thus, of six mice injected with a portion of brain stored for 160 days, one was dead on the fifth day, one was dead and one dying on the seventh, one was dying on the eighth day, one was dying on the tenth and one remained well. The diagnosis of yellow fever infection was made by pathological examination of the brain as well as passage to other mice.

PRESERVATION OF THE MOUSE VIRUS IN 50 PER CENT. GLYCERINE

Infective brain stored in 50 per cent. glycerine at a temperature of 2°-4° C. proved infective when tested after fifty-eight days, but not after 100 days. Under these conditions the brain had lost a good deal of virulence even after fifty-eight days. Only four mice out of six became infected, and two remained well. Three of the mice which became infected did so after a rather prolonged incubation period (eight to ten days). Two brains examined pathologically showed the typical encephalitis and one of these brains proved positive when tested for infectivity.

It is significant that an infective brain stored at 2°-4° C. for fifty-three days in saline proved just as infective as the glycerinised brain mentioned above. Brain in saline kept at 2°-4° C. for 100 days did not prove to be infective.

THE PRODUCTION OF IMMUNITY IN MICE AGAINST AN INTRACEREBRAL INJECTION OF VIRUS

The production of passive immunity by the injection of immune serum produced an immunity in very few cases. Intraperitoneal injection of large quantities ($\frac{1}{2}$-1 c.c.) of human or rhesus immune serum seldom protected against an intracerebral injection of virus, whether tested immediately after the injection of serum or after a lapse of one to eleven days.

The injection of an immune serum intracerebrally on one day and the injection of virus by the same route the next, did not protect any of six mice, though four lived for one day longer than the controls. Mice which have survived an intracerebral injection of a mixture of immune serum and virus, are as a rule just as susceptible as normal animals. In exceptional cases an immunity has been produced this way.

By the intracerebral injection of a sublethal dose of virus, an immunity was occasionally produced. This occurred on two occasions in titration experiments. Of the mice which had survived the injection of diluted virus, several which had received the lowest dilution were found to have become immunized.

Intraperitoneal injection of virus leads to an immunity in about

two-thirds of the mice injected. As pointed out above, the development of immunity after an intraperitoneal injection of virus is not dependent on the dose of the virus.

There are certain indications that the virulence of the virus for mice may have a relation to the production of immunity. Thus, mice injected intraperitoneally with large amounts of virus derived from monkeys, or slightly attenuated mouse virus, such as can be produced by glycerinization or freezing, in no case developed an immunity to a subsequent intracerebral injection of passage virus. The experiments bearing on this point, however, are too few in number to warrant any conclusions.

VIRULENCE OF MOUSE VIRUS FOR RHESUS MONKEYS

The pathogenicity of the mouse virus was tested on three different occasions. In each case the amount injected was approximately the same, namely the entire brains of three mice ill or dying of the infection. The first monkey was injected intraperitoneally with the third passage twenty-four days after the virus had been established in mice. This monkey was dying on the fifth day and was etherized. Autopsy revealed the typical lesions of experimental yellow fever and the diagnosis was confirmed by the microscopic examination of sections.

The second monkey was injected with virus from three mice dying after the twenty-ninth passage, 189 days since the establishment of the virus in mice. This monkey had been used before but there was every reason to believe that he was normal. He showed a temperature on the sixth and seventh days of 104·8° F. and 104·2° F., and thereafter was normal. Twenty-two days after the injection of mouse virus this monkey was given an immunity test with virulent monkey yellow fever virus. He showed a rise of temperature to 104·2° F. on the third day and thereafter was normal.

The third monkey was injected with material from the forty-second passage in mice 273 days after the establishment of the mouse strain. There was no rise of temperature and the monkey remained well until the forty-seventh day, when he appeared to be ill. He was found dead on the morning of the next day. The autopsy findings were consistent with, though not typical of experimental yellow fever in monkeys. There was no jaundice. The lymphatic

glands were enlarged and appeared haemorrhagic. The liver was yellow and fatty, but did not have the typical boxwood colour. The left lung showed a massive pneumonic process.

Microscopical examination of the liver showed a fatty infiltration, particularly in the periphery of the lobules. There were occasional necrotic cells scattered throughout the organ. No intranuclear changes such as have been described as inclusions by Torres (1929) and Cowdry and Kitchen (1929) were found. The appearance of the liver changes are not typical of yellow fever but this disease cannot be excluded.

Mice injected intracerebrally with material from this monkey (blood and liver) either died from a bacterial infection or remained well. Passage to other mice did not result in any infection. All the mice which survived the injection of monkey material, as well as those used in subinoculations, were later given an immunity test with passage mouse virus and all died in the regular time. All the evidence, therefore, pathological and experimental, tends to indicate that this monkey did not die of yellow fever, but of some inter-current infection.

The number of monkey experiments are obviously too few to be of much significance. On account of the fact that one of the monkeys had been used before, only two are of any value. The only legitimate interpretation that can be drawn from the death of the first monkey, is that some of the original yellow fever virus was carried through three mouse passages, and not that proliferation of the virus in the mice had occurred. The development of immunity in the second monkey is significant, though here one cannot be sure that the immunity was produced by the injection of mouse virus. There was every reason to believe that this monkey, though used before, was normal. Assuming that the virus in mice is yellow fever, and the serum experiments leave very little doubt that this is actually the case, the fact that the third monkey lived for forty-seven days after an injection of mouse virus, whether he died from yellow fever or not, warrants the conclusion that continuous passage of the virus through mice leads to an attenuation for monkeys. This opens the interesting speculation whether continuous passage of yellow fever virus through monkeys or mice would also lead to an attenuation for man.*

ACTION OF IMMUNE MONKEY SERUM ON MOUSE VIRUS

In the first experiments to determine the action of immune monkey serum on mouse virus, several procedures were undertaken which are, briefly, as follows :—

1. Three mice were given an intraperitoneal injection of ½ c.c. of immune serum ; a half-hour afterwards they were given an intracerebral injection of virus (brain from twentieth mouse passage). Two of these were in a moribund condition on the eighth day, and one survived. That these two were dying as a result of infection of the virus was shown by microscopic examination of sections of their brains.

2. Four mice were injected intracerebrally with a mixture of equal parts of immune serum and virus. The injections were made immediately after mixing. Two of these were dead on the tenth day and two lived.

3. Three mice received an injection intracerebrally of the immune serum-virus mixture which had been allowed to stand at room temperature for one hour. One was found dead on the ninth day and two survived.

Four mice which received virus alone were all dead or dying on the eighth day. Summarizing the above, of ten mice injected with immune serum and virus, five died and five were protected.

In a second experiment the procedure was somewhat modified. The virus was prepared as usual, then centrifuged at low speed for ten minutes, and the supernatant fluid used. Equal parts of immune serum and virus (brain from thirty-first mouse passage) were thoroughly mixed and allowed to stand at room temperature. After twenty minutes, one hour and two hours' standing, mice were injected intracerebrally with the mixture. A similar mixture was prepared, using, however, normal monkey serum. An equal number of mice was injected at the above intervals. All the mice which received the mixture of normal serum and virus were dead or in a dying condition by the eighth day. The results with the immune serum were as follows :—

1. Of four mice injected intracerebrally twenty minutes after mixing the immune serum and virus, one was moribund on the twelfth day and was etherized, and three remained well.

2. Of four mice injected intracerebrally with immune serum and virus allowed to stand one hour, one died on the third day and three remained well.

3. Of four mice injected with immune serum and virus allowed to stand for two hours, one died on the fifth day, one was dying on the seventh day and was etherized, and two remained well. The mouse that was etherized showed the typical histological picture of yellow fever in mice.

It will be seen from the results of this experiment that the length of time of contact between the immune serum and virus had no marked effect. Of the two mice which died after receiving the mixture of virus and immune serum which had been in contact for two hours, one which died on the fifth day in all probability died as a result of some intercurrent infection, as all the controls at this time were alive and well. The same holds true for the mouse which died on the third day after receiving the mixture which had been allowed to stand for one hour. Excluding these two, the result of the experiment shows that of ten mice injected intracerebrally with a mixture of equal parts of immune serum and virus, two died and eight were protected. Twelve control mice which received mixtures of normal monkey serum and virus intracerebrally were all dead or dying on the eighth day.

The results obtained in this experiment are more clear-cut than those of the first experiment, due no doubt to the use of the centrifuged virus. The method of preparation of the virus in the first experiment was to make a fairly dilute suspension of infective brain, according to the method outlined under the heading ' Technique ' earlier in this paper. It is clear that in such a suspension there must be fairly large masses of brain tissue into the interior of which it is difficult for the immune serum to penetrate and thus to neutralize the virus. It was to overcome this difficulty that the brain suspension was centrifuged in the second experiment.

All the mice in the two above experiments which survived were given an immunity test by the injection of passage virus intracerebrally and all died in the same length of time as the normal controls.

ACTION OF IMMUNE HUMAN SERUM ON MOUSE VIRUS

(A) *Laboratory Infection.*

The serum ' T ' employed in this experiment was that of a laboratory worker who, during the course of these experiments, developed an attack of fever which was at first thought to be a relapse of malaria. This illness was characterised by an irregular temperature ranging from 101° F. to 103° F., and lasting for five days ; a trace of albumin in the urine ; a pulse slow in proportion to the temperature ; a low blood pressure and a marked leucopenia. The source of infection was in all probability from the mouse virus in about the thirty-second passage, as there was very little chance of acquiring the infection in any other way.

Serum taken a week before onset of the illness was available as control to the convalescent serum. The procedure adopted was the same as that of the second experiment above, except that in the preparation of the virus the infective brain suspension was centrifuged at low speed for twenty minutes. Virus (brain from fortieth mouse passage) and sera were mixed and injected immediately into the brains of mice. The results were as follows :—

1. Of five mice which received the mixture of serum obtained before the illness and virus, all were dead or dying by the seventh day.

2. Of six mice injected with the mixture of convalescent serum and virus, one died on the second day, one died on the tenth day and four remained well.

3. Of five mice injected with a mixture of immune monkey serum (also used in the two experiments reported above) and virus, two died on the tenth day and three remained well.

If we exclude the mouse which died on the second day it will be seen that of five mice which received the mixture of convalescent serum and virus, four were protected and one died after a rather prolonged incubation period.

(B) *Convalescent serum from a naturally acquired case in Liberia*

The serum of this patient, ' V,' who had passed through an attack of yellow fever in Liberia, West Africa, was tested in the same manner as described above. The virus (brain from fifty-third mouse passage) was centrifuged for thirty minutes, and equal parts of serum

and virus were mixed and injected immediately into the brains of six mice. Similar mixtures were prepared with ' T ' serum and normal human serum, and each injected into six mice. The results were as follows :—

1. Six mice injected intracerebrally with a mixture of normal serum and virus were all dead on the sixth day except one which, by accident, received a very small injection and which died on the seventh day.

2. Of six mice injected intracerebrally with a mixture of serum ' V ' and virus, one died on the fifth day, one died on the sixth day, one died on the eighth day, one died on the tenth day and two remained well.

3. Of six mice injected with a mixture of ' T ' serum and virus, one died on the second day and one on the third day (both probably due to intercurrent disease), one died on the eighth day, two died on the eleventh day, and one remained well.

It is obvious that in this experiment the virus used was rather virulent, as manifested by the death on the sixth day of all the mice which received the mixture of normal serum and virus, and by the survival of only one mouse which received the mixture of ' T ' serum and virus. In repeated neutralization tests with one serum and various preparations of virus obtained from different mice, the number of mice which survived varied, due probably to the difference of virus content in the mouse brains used.

Protection tests similar to the above have been carried out with convalescent yellow fever sera from Africa, Brazil and Colombia, and all with possibly one exception have shown some protection. It is hoped that the results of these tests, some of which are still in progress, will be published in a separate article in the near future.

GROSS PATHOLOGY

Mice dying after an injection of yellow fever virus show very few macroscopic changes. Many appear to be perfectly normal. There is no jaundice. One lesion, namely, haemorrhage into the stomach, seems to be common enough to suggest that it is due to the action of the virus. The stomachs of mice dying of yellow fever are almost invariably empty and in about half of the cases show definite

haemorrhages. The blood in the stomach becomes black and tar-like, presenting the same appearance as that seen in monkeys dying of infection with the yellow fever virus. No definite ulceration has been found, nor has microscopic examination of sections revealed the genesis of these haemorrhages.

A large proportion of the mice show changes in the lymph glands, best seen in the inguinal region. The glands are usually enlarged and hyperaemic, and sometimes there is a distinct haemorrhage into the substance of the gland as well as into the surrounding connective tissue. Small subcutaneous haemorrhages are sometimes seen in the axillae or on the abdominal wall. However, similar changes in the lymph glands have been observed in the stock mice. The appearance of the abdominal organs is usually within the normal range, such pathological changes as are sometimes seen being due to other infections such as mouse typhoid. The liver sometimes appears markedly fatty but this condition one also sees in stock mice. Sections of such livers show a fatty infiltration of the liver cells, but no necrosis.

Mouse typhoid was fairly often encountered in our injected mice. This infection may cause haemorrhages into the intestine as well as into the stomach. In such cases the stomachs resemble very closely those of yellow fever mice. We had thus considerable doubt at first whether any of the gastric lesions were associated with infection with the virus of yellow fever.

MICROSCOPIC PATHOLOGY

On account of the sharp localization of the virus of yellow fever in the nervous system of an infected mouse, attention was chiefly directed to the study of changes in the brain. Sections of this organ showed an encephalitis which in well-marked cases is reminiscent of that seen in encephalitis lethargica. These lesions consist of a proliferation of the vascular endothelium and a peri-vascular infiltration with mononuclear cells. The lesion is in some instances of the type commonly designated perivascular endothelial proliferation. The vascular changes are first seen in animals killed on the third day after injection and become more marked on the succeeding days until the death of the animal. In mice which, for

some reason or other, live longer than usual the pathological changes are usually correspondingly more marked than in those which die in the regular time.

In addition to the vascular changes there are almost invariably present nuclear changes in the ganglion cells which resemble those observed in the nuclei of liver cells of rhesus monkeys infected with the virus of yellow fever and which have been described as inclusions by Torres (1929), and Cowdry and Kitchen (1929). These specific nuclear changes take the eosin stain intensely with the eosin-methylene blue method and are coloured pink with Giemsa's stain. They are particularly abundant and well-marked in young mice dying after an intraperitoneal injection of virus. When they are well developed the nucleolus is seen to be almost completely sur-rounded by an eosinophilic body consisting of a large number of granules. It is fairly frequently observed that in addition to the nuclear changes the cytoplasm of the cell takes on an eosinophilic tint. This alteration in the staining reaction appeared in some cases at least to be due to the presence of vast quantities of small acidophilic granules. Vascular as well as nuclear changes are also observed in the spinal cord.

The encephalitis as well as the nuclear changes have been observed in the brains of mice injected with yellow fever material from three different monkeys and are present no matter how the mice are infected. However, a few brains have failed to show the specific · nuclear changes even after repeated examinations.

Since the above was written a second and more lengthy account by Cowdry and Kitchen (1930) of the nuclear changes which occur in yellow fever livers of man and monkeys has appeared. From the detailed descriptions and illustrations by these authors I have no hesitation in stating that the appearance as well as the development of the nuclear changes in the ganglion cells in infected mice are very similar to those in the nuclei of the liver cells of man and monkey.

DISCUSSION

The neurotropic character of yellow fever virus in mice is strong evidence in favour of the theory that the etiological agent of this disease should be classified with the filterable viruses. As far as

I know, no one has shown any tropism of yellow fever virus in man or monkey. The affinity of a virus for a definite tissue in one susceptible animal and not in another is a rather unusual occurrence. The closest analogy is afforded by the virus of fowl plague (Geflügelpest). Fowl plague in chickens produces an acute disease with the virus distributed throughout the animal : blood, abdominal organs, and nervous system. Adult geese as a rule are not susceptible to a subcutaneous or intramuscular injection of fowl plague virus, but can be infected by an intracerebral injection (Kraus and Schiffmann, 1907). According to Kleine (1905) young geese are susceptible to a subcutaneous injection of fowl plague virus. He could demonstrate the virus in the blood up to four days after injection when it disappeared. It could then be demonstrated in the brain, spinal cord, and sciatic nerve. Kleine, and independently of him, Rosenthal (1906) compared the virus of fowl plague to rabies. Neurotropic properties of fowl plague virus in pigeons have been also shown (Kraus and Doerr, 1908).

SUMMARY

Mice were injected intracerebrally with yellow fever virus from four different rhesus monkeys. In every experiment some of the mice became ill and died. The virus can be propagated indefinitely in mice by mouse brain to brain inoculations.

The virus of yellow fever is highly neurotropic in mice, being present at death in high concentration in the brain, spinal cord, sciatic nerve and adrenal. The virus is either entirely absent, or is present in very small amounts in the blood, liver, spleen, kidney, and testis.

Injection of the virus into the nervous system, e.g. eye, brain, spinal cord, results in infection. Injection of the virus subcutaneously, intradermally, intramuscularly, intraperitoneally, very seldom indeed results in infection and death but often produces an immunity to a subsequent intracerebral injection of passage virus.

Evidence is presented that in mice the virus travels along the nerve paths, both centripetally and cetrifugally.

Young mice less than two weeks of age are susceptible to an injection of the virus into the subcutaneous tissues and the peritoneal

cavity as well as to intracerebral injection. The distribution of virus in the body of a young mouse dying after an intraperitoneal injection is the same as that in an adult mouse after an intracerebral injection—viz., the nervous system is highly infective, whereas the blood and abdominal organs (with the exception of the adrenal) are either free from virus or contain very little. Virus injected into the abdominal cavity of a young mouse cannot be demonstrated in the blood or abdominal organs (liver, spleen, kidney) forty-eight hours after the injection. The brain at this time is already highly infective.

The serum of a monkey immune to yellow fever, human serum from a laboratory infection as well as human sera from naturally acquired infections neutralize the mouse virus partially or completely. This effect can be shown by mixing the immune serum and virus *in vitro* and then injecting the mixture intracerebrally into mice.

Infective mouse brain stored in the freezing room at − 8° C. retains its virulence for at least 160 days. Kept in 50 per cent. glycerine at 2°-4° C. brain proved infective after fifty-eight days but not after 100 days.

Passage of the yellow fever virus through mice leads to a loss of virulence of the virus for monkeys. A rhesus monkey injected with the third mouse passage died of typical experimental yellow fever in five days. A monkey injected with material from the forty-second mouse passage remained well for forty-seven days. This monkey was found dead on the forty-eighth day—death in all probability being due to intercurrent disease.

The brains of all mice, no matter how infected, show an encephalitis which in well-marked cases is highly reminiscent of encephalitis lethargica. In addition to the vascular changes there are present almost invariably nuclear changes in the ganglion cells, which resemble those present in human and monkey yellow fever livers and which have been described as inclusions by Torres (1929) and Cowdry and Kitchen (1929).

CONCLUSIONS

1. Mice are susceptible to the virus of yellow fever when injected into the brain. The strain can be maintained indefinitely by brain to brain passages.

2. The virus established in mice is highly neurotropic for these animals.

3. The action of immune yellow fever sera can be demonstrated by injecting a mixture of immune serum and virus into the brains of mice.

4. Passage of yellow fever virus through mice leads to a loss of virulence for monkeys.

5. Yellow fever virus produces an encephalitis in mice. In addition there are specific eosinophilic nuclear changes in the ganglion cells.

6. The neurotropic character of the virus as well as the pathological changes induced, afford strong evidence that the etiological agent of yellow fever should be classified with the filterable viruses.

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EXPERIMENTAL STUDY ON THE RESIST-ANCE OF *ENTAMOEBA HISTOLYTICA* TO EMETINE HYDROCHLORIDE *IN VITRO*

BY

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The following experiments were made in an endeavour to see how far it is possible to make a strain of *Entamoeba histolytica* resistant to the action of emetine hydrochloride in culture tubes. With this end in view the following four strains of the parasite were isolated.

STRAIN V. This was isolated from a patient suffering from chronic intestinal amoebiasis, who was passing cysts in his stools at the time when the specimen was taken. When the specimen of faeces was obtained it was first microscopically examined. All the cysts found were those of *E. histolytica*; there were no amoeboid forms or other protozoa to be detected. A suspension of cysts was made, using the concentration method of Yorke and Adams.

The differential count of the cysts was as follows :—mono-nucleated 56 per cent., binucleated 20 per cent., quadrinucleated 22 per cent., and unclassified cysts 2 per cent.

From this suspension three tubes of Boeck and Drbohlav's medium, and three tubes of Dobell's medium were inoculated ; sub-culture was carried out every alternate day. After the strain had been established it was tested on emetine media of various concentrations as shown in the tables below.

STRAIN G. This strain was isolated from a patient with the following history :—He first had an acute attack of diarrhoea in 1916, when he was admitted to Hospital in Nairobi for eight days ; in 1917 he had a second attack. Specimens examined then were said to be negative for *E. histolytica*. In March, 1928, a third attack of acute diarrhoea was accompanied by passage of blood

and mucus in the stools. The faeces on examination was then found to be swarming with vegetative forms of *E. histolytica*. A course of emetine was given, amounting to 10 grains. In July, 1928, there was a relapse, when 8 grains of emetine were administered; in November, 1928, 6 grains of emetine were given during an attack. In July, 1929, the latest attack began but no emetine was given until after isolation of the strain—in August. The strain was isolated from the faeces of the patient on two different occasions, not during an acute attack, but in the intervals; the first occasion being on 1.8.1929, and the second on 26.8.1929. During the period elapsing between the two mentioned dates there had been no emetine administered.

STRAIN GI was the name given to that isolated on the second occasion. These strains were established on egg-solid and Ringer's egg-white solution.

STRAIN MENA. Strain Mena was obtained from a patient who contracted amoebic dysentery during the war, in 1917; since then he had had several acute relapses. The patient was under the care of Professor Yorke when the strain was isolated; he was then having an acute relapse and passing blood and mucus. The macroscopical appearance of the stool was that very characteristic so-called ' red-currant jelly.' Microscopically it was teeming with vegetative forms of *E. histolytica*. The individuals were strikingly large in size, measuring from 20μ to 30μ, and the majority of them had ingested numerous red blood corpuscles. This strain was isolated on a medium composed of egg solid, and Ringer's egg-white solution.

The technique adopted in carrying out the experiments mentioned below consisted of preparing egg slopes as described by Boeck and Drbohlav, and the addition of egg-white and Ringer's solution (white of one egg to 250 c.c. of Ringer's solution). The emetine media were prepared by adding emetine hydrochloride to both solid and fluid parts in the same concentrations in each tube. Each tube contained 5 c.c. solid and 8 c.c. fluid. In every experiment the tubes were put into the incubator for one hour before use, in order to warm them, a little rice starch was added and then they were inoculated from the stock cultures of the various strains above mentioned.

It was first thought necessary to discover the concentration of
emetine which would kill *E. histolytica in vitro* incubated at 37° C.
in a given time.   This was done by examining the emetine cultures
after forty-eight hours, and then again after seventy-two hours
from the time of incubation.   In Table I are indicated the number of
cultures which grew and the number which died in the concentrations
given.   All control cultures showed good growth.

TABLE I.

Showing growth of the four strains in various emetine concentrations.

Concentration	Strain V		Strain G		Strain G1		Strain Mena	
	Grew	Died	Grew	Died	Grew	Died	Grew	Died
1 : 1,000	o	3	–	–	–	–	–	–
1 : 10,000	o	3	–	–	–	–	–	–
1 : 20,000	o	3	–	–	–	–	1	3
1 : 40,000	o	3	o	3	o	4	4	o
1 : 50,000	–	–	–	–	1	3	4	o
1 : 80,000	o	3	1	2	4	o	4	o
1 : 160,000	3	o	3	o	4	o	–	–
1 : 320,000	3	o	3	o	–	–	–	–
1 : 640,000	3	o	3	o	–	–	–	–

NOTE.—o = Nothing grew.   – = No tube inoculated.

In Table II are given in detail the results obtained by submitting
Strain Mena to the following concentrations of emetine hydrochloride.
The technique used here was the same as employed in the three
previous strains.

TABLE II.

Showing concentrations used and the results.

Concentration	Grew	Died
1 : 20,000	1	3
1 : 30,000	3	1
1 : 40,000	4	o
1 : 50,000	4	o
1 : 80,000	4	o
1 : 100,000	2	o
1 : 120,000	2	o
1 : 160,000	2	o
1 : 200,000	2	o
1 : 400,000	2	o

NOTE.—All control tubes showed very copious growth.

The results of the experiments which are shown in the Tables No. I and No. II led me to draw two conclusions. *Firstly ;* The threshold lethal concentration of emetine for *E. histolytica* differs in each of the four strains. *Secondly ;* The strain which is most resistant to emetine is that—Mena Strain—isolated from a case subject to acute relapses, with passage of blood and mucus in loose stools swarming with large vegetative forms containing numerous ingested red blood corpuscles.

The reproductive power of *Entamoeba histolytica* is not impaired by emetine of a concentration of 1 in 160,000. An amoeba of Strain G in this strength was observed during process of division ; the movements of the two resulting daughter amoebae were observed for some time, and they were apparently quite healthy. As it was noted that G Strain was able to resist higher concentrations of emetine than V Strain, it was subjected to the following experiments. The sub-culture of 1 in 160,000 was sub-inoculated into media of the same concentration every third day for five successive generations. The growth in the second generation was far better than that in the first, but in the fourth and fifth, especially in the latter, there was produced a small race measuring from 12μ to 14μ, whereas the original G amoebae measured from 18μ to 23μ. No cysts were seen during any of these experiments. By growing this small race on normal medium there was obtained a good growth of amoebae of the same size and quality as the original G. On inoculating a tube of 1 in 80,000 with the small race of the fifth generation in 1 in 160,000 a very scanty growth was to be observed after three days' incubation. On transferring material from this tube, however, to a tube with normal medium, a good growth of amoebae occurred.

The behaviour of the Strain G1 differed in no great extent from that of its predecessor, Strain G. Both had an average measurement of 18μ to 23μ.

Strain Mena was no doubt as shown in the Tables Nos. I and II, much more resistant to emetine than the other strains examined. As already noted, the faeces from which it was isolated contained an abundance of vegetative forms of *E. histolytica*, and the individuals varied in size from 20μ to 30μ, and contained numerous ingested red blood corpuscles. In cultures, the amoebae showed great ability for

ingesting starch, some individuals would ingest as much as ten granules, so as to have a packed appearance. After adding a drop of blood to cultures of this strain some amoebae were seen with ingested erythrocytes. This strain which grew copiously on emetine free media, could be maintained easily in cultures, and was free from admixture of other protozoa as proved by numerous tests. It was selected and subjected to the following experiments in order, if possible, to raise its resistance to emetine.

The technique used for these experiments differed in the following points from that used for obtaining the above-mentioned results. Two media were used here ; the first was a complex medium composed of inspissated ox-serum as solid and 8 c.c. of Ringer's egg-white solution for the fluid part ; a few grains of starch were added to each tube. The emetine was mixed with the fluid part of those tubes which were used for graduating the amoeba to raise its resistance to the alkaloid. To obtain results as uniform as possible, the inspissation of the serum was carried out with great care, in order to get each batch of medium of the same clarity as the previous ones. This was done by keeping several tubes as standards for comparison.

The examinations of the tubes were done after incubating them at 37° C. for three days. The deposit in the tubes was microscopically examined, and in doubtful cases the deposit was mixed thoroughly with all the fluid in the tubes and centrifugalised, and then examined. Sub-inoculations on normal media were also done in some cases to obtain decisive results. The negative tubes showed either nothing at all, or some dead amoebae were encountered during examination. The dead amoebae had a granular appearance ; the nucleus (in fresh specimens) was sometimes very distinctly visible with chromatin dots along the nuclear membrane ; sometimes they showed large vacuoles where the cytoplasm had been dissolved.

As shown in Table III, col. 1, below, the first concentration of emetine used was 1 in 400,000, and this was raised gradually. Whenever the strain during its graduation in emetine showed signs of deterioration it was returned to weaker concentrations, and then once again taken through the higher concentrations, but it was never allowed to touch emetine-free media. These experiments were carried on for five months, and at last a strain was obtained which

showed a definite resistance to emetine hydrochloride as compared with amoebae from the original strain which was grown on normal media and never treated with emetine *in vitro*.

TABLE III.

Showing growth of Mena Strain under various experimental conditions.

Concentrations of Emetine hydrochloride		Growth of Mena Strain in successive passages in Emetine medium	Test of Normal Mena Strain inoculated directly into Emetine medium	Growth of Mena Strain in Emetine medium 1 : 140,000 and progressive passages
	Generation			
1 : 400,000		+++	...	...
1 : 200,000		+++	...	...
		(measurement 18μ-30μ)		
1 : 160,000		+++	...	...
1 : 120,000		+++	...	...
1 : 100,000		+++	...	...
1 : 80,000		+++	...	...
1 : 50,000		++	...	...
1 : 40,000		+	...	...
1 : 30,000	I	+	...	...
1 : 30,000	II	++	...	...
1 : 20,000	I	++	...	...
1 : 20,000	II	++	...	...
1 : 20,000	III	+	−	...
		(cysts were present)		
1 : 20,000	IV	+−	...	...
1 : 40,000		+	...	...
1 : 80,000		+++	...	...
1 : 50,000		+++	...	...
		(measurement 23μ-30μ)		
1 : 40,000		+++	...	...
		(measurement 14μ-28μ)		
1 : 30,000		++	...	...
1 : 20,000		++	...	...
1 : 50,000		+++	...	...
1 : 20,000	I	++	...	...
1 : 20,000	II	+	...	...
1 : 20,000	III	++	...	...
1 : 20,000	IV	+	...	...
1 : 20,000	V	++	...	...
		(measurement 14μ)		
1 : 20,000	VI	+	...	...
1 : 50,000		++	...	...
1 : 20,000		+−	...	...
1 : 200,000	I	+++	++	...
1 : 200,000	II	+++	...	...
1 : 180,000		+++	...	....
		(measurement 18μ)		
1 : 160,000		+++	...	...
		(measurement 20μ)		
1 : 140,000		+++	...	+++
1 : 120,000		+++	...	++
		(few cysts present)		
1 : 100,000		+++	...	++
1 : 80,000		+++	...	+++

TABLE III—*continued*.

Shewing growth of Mena Strain under various experimental conditions.

Concentrations of Emetine hydrochloride		Growth of Mena Strain in successive passages in Emetine medium	Test of Normal Mena Strain inoculated driectly into Emetine medium	Growth of Mena Strain in Emetine medium 1 : 140,000 and progressive passages
	Generation			
1 : 70,000		+++	...	++
1 : 60,000		+++	++	+++
1 : 50,000		+++	...	+
1 : 40,000		++	−	+
1 : 30,000	I	++	−	+−
1 : 30,000	II	++	+−	+
1 : 20,000	I	++	+−	++
1 : 20,000	II	++	Tube No. 1 − ⎫ ,, No. 2 − ⎬	−
1 : 20,000	III	++	+−	−
1 : 15,000	I	++	Tube No. 1 − ⎫ ,, No. 2 − ⎬	...
1 : 15,000	II	+ (measurement 8μ-14μ)	,, No. 1 − ⎫ ,, No. 2 − ⎬ ,, No. 3 −	...
1 : 50,000		++ (measurement 14μ)	+ (measurement 18μ)	...
1 : 30,000		++ (measurement 14μ)	−	...
1 : 20,000	I	++	...	...
1 : 20,000	II	++	...	...
1 : 10,000	I	++	−	...
1 : 10,000	II	+++	−	...
1 : 10,000	III	++	−	...
1 : 10,000	IV	++	...	...
1 : 10,000	V	++	..	..

The first column of Table III shows the various concentrations through which this strain was passed from one to the other. The second column indicates the growth results obtained during these passages. The third indicates the results obtained in a series of inoculations of amoebae from emetine-free cultures into a medium containing the given concentrations of emetine. The fourth column gives the results of inoculating at 1 in 140,000 concentration of emetine medium, the same Mena Strain and attempting to carry it on from this to higher concentrations by direct passage without any intermission. It will be seen that it does not survive a concentration of 1 in 20,000. This may be attributed to the fact that it has had a shorter exposure previously to emetine graduations and also that it was not brought back into weaker concentration at the first signs of failure to grow.

The table shows that while amoebae from emetine-free cultures could not survive at all in the concentrations of 1 in 40,000, 1 in 30,000 and 1 in 20,000, or could yield only a very poor growth, the emetine-graduated amoebae could resist stronger concentrations (1 in 20,000, 1 in 15,000, 1 in 10,000) and could easily be maintained in them for several generations, and yield copious growths. The measurements of the emetine-graduated amoebae were taken at intervals (as given in the Table III in the second column) and showed a marked reduction in size, which they had undergone by passage from lower concentrations to higher ones.

The second medium was a fluid medium and was used in order to confirm the results shown in Table III on the emetine-graduated strain. This simple fluid medium permits the action of emetine on the amoeba in much greater dilutions than do the complex media. For example, the Mena Strain died in a concentration of 1 in 200,000 in a few days. White of one egg was mixed with 250 c.c. of Ringer's solution. It had been found by previous experiments that on such a medium, amoebae from an already established growth could survive for two generations, yielding good growths.

The experiments shown in Table IV were then performed as follows :—Small tubes, 7·5 cm. in length and 12 mm. in width, were used here. Each tube received 5 c.c. of the fluid medium mixed with the required concentration of emetine. Tubes No. 1 were inoculated from the emetine-graduated strain, and Tubes No. 2, containing the same concentrations of emetine, were inoculated from the cultures which had never been treated with emetine. Controls (Tubes No. 3), containing no emetine, were inoculated from normal cultures of Mena Strain.

As shown in Table IV, Tubes No. 2 were found to be either negative or showing very few amoebae on verge of death, in a concentration of 1 in 200,000 after two days' incubation.

On the other hand, the amoebae of the emetine-graduated strain, Tubes No. 1, was able to survive in a concentration of 1 in 50,000 for two days. This means that the emetine-graduated strain could tolerate a concentration which is four times as much as that sufficient to kill the amoeba from normal cultures.

TABLE IV.

Showing the different behaviour of mormal and Emetine-graduated Strains in an emetine fluid-medium.

Concentration of emetine hydrochloride	Number of days of incubation	Tubes No. 1		Tubes No. 2	
		These tubes were inoculated from the emetine-graduated Strain		These tubes were inoculated from normal Mena Strain	
		Survived	Died	Survived	Died
1 : 500,000	1	2	0	2	0
	2	1	0	1	0
	3	1	0	1	0
	4	1	0	1	0
1 : 200,000	1	1	0	1	0
	2	2	0	1	1
	3	3	2	0	5
1 : 100,000	3	1	0	0	1
	4	1	2	0	3
1 : 50,000	2	3	1	0	4

Tubes No. 3.—Twenty Tubes contained the same medium without emetine, and were inoculated from the stock cultures of normal Mena Strain. These were used as controls, and all showed good growth of amoeba.

CONCLUSIONS

1. The resistance of *Entamoeba histolytica* to emetine hydrochloride can be raised by passing it systematically for a long period through graduated concentrations of that drug.

2. The amoeba undergoes a reduction in size after its passage over a long period through emetine hydrochloride.

3. The resistant amoeba retains its reproductive powers.

DISCUSSION

Vedder, in 1911, was the first to discover the powerful action of emetine on free-living amoebae. Rogers (1912) used emetine injections hypodermically in the treatment of severe cases of amoebiasis.

Since then many workers have tested the efficacy of emetine in the treatment of infections with *Entamoeba histolytica*. Baermann

and Heinemann (1913) state that after a period of about ten days after cessation of the treatment, the amoebae tend to reappear in the stools. Walker (1913) writes : ' None of the twenty experimentally infected or the nine naturally infected men has ceased to be a carrier of the *Entamoeba histolytica,* although some of them have been under observation for two years.' Whitmore (1914) notes that relapses are not uncommon. Phillips (1914) states that emetine often fails to eradicate the infection. Sandwitch (1914) recorded fatal cases in spite of emetine treatment. Chalmers and Archibald (1915) point out cases of infections of prolonged duration up to three years or more. Barlow (1915) states that his patients who were treated for only three to six days with emetine, all relapsed. J. G. and D. Thomson (1916) expressed their opinion that insufficient treatment with emetine is dangerous as it converts the patient into a carrier. Dobell (1916) concludes that emetine hydrochloride given hypodermically in small quantities very rarely rids a carrier of *E. histolytica* infection. He states further that full courses of the drug (10 to 12 grains or more) are successful in about one-third only of the cases treated. Re-treatment with equal or larger amounts of the drug, of patients who have already received full courses of emetine offers little hope of success. Jepps (1916), Job and Hirzmann (1916) recorded similar results.

Craig (1917) states that the drug has wonderful effect on the acute symptoms, but even if it is administered until toxic symptoms appear, it does not actually cure infection or prevent carriers, save in a very few cases. Wenyon and O'Connor (1917), after conducting the treatment of a great number of cases in Alexandria, come to the conclusion that the cases which are most difficult to rid of infection are not the carrier cases passing cysts without symptoms, but the acute cases passing blood and mucus in the stools with active amoebae containing erythrocytes. These acute cases have nearly all had a history of recurrent attacks of dysentery and generally previous emetine treatment. On the suggestion that the amoebae may acquire the power of resisting emetine, they comment in the following passage :—' It is very difficult to obtain precise information on this point, for, as already explained, the failure of emetine to cure may be due purely to mechanical reasons of poor circulation or other causes.' They, on the other hand, give a record of ' a liver abscess in

which in spite of treatment by a full course of emetine injections, and irrigations of the abscess cavity, amoebae were constantly detected in the pus.' Amoebae from this pus were mixed with an emetine solution and watched by them under the microscope. The amoebae moved actively in the liquid and only came finally to rest after the expiration of ten minutes. They state that the emetine solution in which the amoebae were moving was many times in excess of the usual 1 in 100,000 which, it is claimed, kills them at once. They express their opinion on this point as follows : ' Whether these amoebae were emetine-resistant or not, cannot be decided till similar observations are made with amoebae from abscess in cases which have not received previous emetine treatment.'

Mayer (1919) states that he has observed emetine-fast races in chronic amoebiasis. Macadam (1918), Hage (1923), Willmore (1923), and Oliver (1924), give records of disappointing results and of a great number of relapses after emetine treatment. Sellards and Leiva (1923) consider that recovery from amoebic dysentery results from the natural resistance of the host and a moderately toxic action of emetine on the entamoebae.

Drake-Brockman (1926) considers that the excessive use of ipecacuanha and its derivatives renders the amoebae emetine-fast. Brown (1928) states that certain cases seem to be very resistant to treatment. Knowles and his co-workers (1928) have published a detailed paper dealing with the whole question of the treatment of Amoebiasis. They, after reviewing the literature on the subject, conclude that the general consensus of opinion appears to be ' (a) that emetine injections are by far the most satisfactory immediate line of treatment for amoebic dysentery, but (b) that emetine therapy is generally a failure in the treatment of the carrier condition.' They write further that : ' It has been shown by S. P. James (1926) that primary malaria, as therapeutically induced in the treatment of general paralysis of the insane, is a disease which is very readily amenable to quinine treatment ; only a few days of quinine treatment are necessary to effect a cure without relapses. On the other hand, the experience of all workers in the tropics is that established and relapsing malaria is very difficult to eradicate. Possibly a similar state of affairs exists with regard to amoebic infection.'

It is interesting to note Plehn's (1927) records of quinine-resistant

strains of the plasmodia of man, and his endeavour to explain how the parasites remain unaffected while the condition of the patient has generally improved. His three explanations are the following :—

' (1) The biological properties of the quinine-resistant parasites will be, through quinine directly or indirectly, so changed that they produce no poisonous metabolic products, while they maintain their vitality and reproductive power,
Or

(2) The action of quinine is not only parasiticidal, but at the same time anti-toxic, i.e., it eliminates the manifestations of the disease not only through its destruction of the parasites, but by means of its direct action on the metabolic products without destruction of the parasites themselves. Parallel to this is the inhibitory action of quinine on certain ferments (lipase and invertase).

(3) The symptoms of the disease are produced not directly by the toxins of the parasites but by the products of the reaction of the human organisms. The quinine. prevents this reaction either by paralysing the products in a similar way, as in the case of the lipase poisoning, or it paralyses the cells which produce these products without however, attacking the quinine-resistant parasites.'

These, of course, as the author states, are mere hypothetical explanations, but in any case they throw light on what might take place when the plasmodiae, amoebae, or trypanosomes acquire certain resistance to drugs.

Here, on the other hand, reference must be made to the experimental work of Dobell and Laidlaw (1926), on the action of ipecacuanha alkaloids on *Entamoeba histolytica* and other entozoic amoebae of man. These authors state that ' Emetine and cephaline have been found to be specific poisons for *Entamoeba histolytica* under natural conditions.' They write further that :

' In another series of experiments an attempt was made to ascertain whether *Entamoeba histolytica* is able to acquire a resistance to emetine by prolonged cultivation in the presence of tolerated doses of the alkaloid. The experiments were made as follows : One of the positive cultures of *E. histolytica* in 1 in 50,000 emetine was taken, and successive sub-cultures were made into a medium containing the same amount of alkaloid. In this way the strain was cultivated with difficulty in 1 in 50,000 emetine for over a month. From time to time during this period, sub-cultures were made into higher concentrations of emetine and if the amoebae survived, an endeavour was made to establish a resistant strain by subcultivation in higher concentrations. Two results emerged from these experiments. First, it was found that continuous cultivation in 1 in 50,000 emetine was not smooth, or easy—growth being irregular and slow. A culture five or six days old was almost inferior to a one-day culture without alkaloid. Secondly, it was found impossible to secure continued growth in concentrations of emetine higher than 1 in 50,000. These experiments, therefore, confirm the conclusions drawn from those previously quoted, and they also indicate that it is not easy—they suggest, in fact, that it is impossible, to obtain a strain of *E. histolytica* which is resistant to emetine.'

Vogel (1927), carrying out a similar work on the action of yatren on *E. histolytica,* *in vitro*, concludes that : (1) Yatren has direct

toxic action on *E. histolytica*. A solution of 1 : 100 kills the amoeba in a few hours ; 1 in 1,000 and 1 in 5,000 inhibit the reproductive power of the parasite and as a result the cultures die. (2) The amoeba could be cultivated with difficulty in 1 in 10,000 for ten generations. (3) The amoeba does not lose its reproductive power if subjected to Yatren solution of 1 in 1,000 for fifteen hours, but if left longer it cannot multiply any more, if inoculated on a normal medium.

In my opinion these authors were endeavouring to prove the absolute toxicity of these drugs employed in their experiments with *E. histolytica*. They also carried out their experiments over a very short time. They have begun with a concentration nearly lethal to *E. histolytica, in vitro*, instead of beginning with very weak concentration and gradually raising it, till finally a very strong concentration is reached.

Here, in this present work it is clearly shown that the amoebae which were graduated systematically through emetine, over a period of nearly five months, were far superior in resisting the drug, to those which had not been cultivated on emetine media at all.

Yorke and Adams (1926) have shown that cysts of *E. histolytica* are remarkably resistant to emetine and yatren. They have also (1927) shown that freshly-passed faeces contain something which inhibits excystation. Therefore the opinion expressed by Mayer (1919), that relapses after treatment may be normally due to the cysts hatching inside the intestine, is hard to accept.

Some authors (Wenyon and O'Connor, 1917, and others) attribute relapses after treatment to mere mechanical reasons such as poor circulation, yet they admit that cases very difficult to rid of infection are those exhibiting acute relapses and passing blood and large vegetative forms of amoebae containing erythrocytes.

Dobell and Laidlaw (1926) were inclined to attribute the incurability of these human cases rather to the peculiarity of the patient than to his parasite. They suggested then a rapid excretion of the drug by the kidney of man similar to that which occurs in the cat, so that little or none of it reaches the bowels. The same authors (1928) state that the amoebicidal efficacy of emetine depends also on the reaction of the medium.

Oliver (1924) states that the treatment of amoebiasis by emetine was more successful in hot weather than in winter.

SUMMARY

The workers on this problem are, and will probably long remain divided in their theories as to the cause of resistant cases. The chief and most feasible of these theories advanced are :—

1. An acquired resistance to emetine by *Entamoeba histolytica* after inefficient treatment.

2. Mechanical causes such as fibrosis around the diseased tissues preventing access of the drug via the blood (Wenyon and O'Connor, 1917, Knowles and his co-workers, 1928).

3. Excystation of the resistant cysts in the bowels after cessation of treatment. (Mayer, 1919.)

4. The hydrogen-ion-concentration plays a rôle in the efficacy of the drug. (Dobell and Laidlaw, 1928.)

5. Rapid excretion of the drug by the kidneys preventing it reaching the site of the lesion, as in the case of the cat. (Dobell and Laidlaw, 1928.)

6. A diminution of the curing properties of the drug during the cold weather (Oliver, 1924) is possibly associated with a change in the endocrine rate of metabolism leading to a loss of resistance on the part of the patient. (Knowles and co-workers, 1928.)

Whatever may be the cause, or combination of causes of the occurrence of cases of amoebic dysentery resistant to emetine in spite of prolonged treatment, the results of my experiments lead me to conclude that one of the chief of these, is a tolerance to the drug gradually acquired by the amoebae. My culture of Mena Strain already showing some signs of resistance higher than the amoebae of the other three strains, was finally exalted through emetine tubes to a resistance four times that of untreated amoebae of the same strain, eight times that of the amoebae of V and G Strains, and five times that of the amoebae of Dobell and Laidlaw (1926). These results shed light on the interesting phenomenon of acquired drug tolerance already known to exist, to even a greater degree, among other parasitic members of the phylum protozoa.

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AN ACCOUNT OF TRYPANOSOMIASIS AT THE CAPE LIGHTHOUSE PENINSULA, SIERRA LEONE

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I. INTRODUCTION AND SUMMARY OF PREVIOUS OBSERVATIONS

The attached map shows that the Cape Lighthouse Peninsula is a mushroom-shaped piece of land lying west of Freetown and comprising an area of about 700 acres. This district, if it were completely free of tsetse, would appear to be an excellent site for the establishment of an experimental cattle and sheep station. Healthy cattle, imported from other countries for breeding purposes, could be safely landed at its northern extremity without the danger of infection usually incurred by an overland journey through districts where fly occur, while its separation from the mainland by the long isthmus would render negligible the danger of its being invaded by ' travelling fly.'

Unfortunately, *G. palpalis* is very prevalent in the area, and it is in connexion with a scheme for ridding the Peninsula of tsetse that various authorities have carried out observations, commencing with Yorke and Blacklock (1915) and continuing until the writers' observations in 1928 and 1929. So far as we are aware, the records quoted are almost the only ones concerning trypanosomiasis in Sierra Leone. These intermittent investigations, which extend over a period of fourteen years, represent a rather more lengthy observation of one isolated area than is usually possible in the tropics and are of special

interest because bush-clearing as an anti-fly measure was in progress during the latter six years. Before detailing our results it appears advisable to give a brief summary of those obtained by previous workers.

1. Yorke and Blacklock (1915a and 1915b). These observers record that a few cases of human trypanosomiasis existed in the area, but they did not personally examine either the human or animal population. They dissected 400 fly and showed that 5·2 per cent. were infected with trypanosomes, the anatomical distribution of infection being 71 per cent. in the proboscis alone, 19 per cent. in the proboscis and gut, and 9·5 per cent. in the gut alone, none being infected in the salivary glands. They showed that the number of fly present at various parts of the Peninsula was considerable, but did not express these results numerically. Their main investigation, carried out during the months December to February, inclusive, concerned itself with a very thorough examination of the breeding places and bionomics of *G. palpalis*. Their results appear to prove conclusively that the breeding places at that time were confined almost exclusively to the shade of the oil palm (*Eloeis guineensis*). At this period of the year they found an average of ten to twenty pupa-cases under every oil palm examined. They recommended that in order to destroy the breeding places of the fly, the lower petioles of the young oil palms should be stripped.

2. Blacklock (1922) studied the same area during July and August. He did not examine the human or animal population but confined his attention to the prevalence of fly, their breeding places, and the proportion of fly infected with trypanosomes. He showed that the number of fly caught per boy per hour was represented by the figure 4·8. The percentage of infected fly was 5·9, as compared with Yorke and Blacklock's figure of 4·7 per cent. for the 1914 investigation. He dissected 471 flies, twenty-eight (5·9 per cent.) of which were infected in the proboscis and one in the salivary glands. Blacklock did not include an examination of the gut in all his dissections and this omission tends to reduce his figure for the percentage of infected fly to a lower figure than was actually the case ; that this discrepancy is negligible is proved from Yorke and Blacklock's (1915b) and our own 1928 and 1929 figures, which show that out of a total of 1,595 fly dissections, only eight (0·5 per

cent.) were infected in the gut alone. Blacklock's (1922) figures for the percentage of fly found infected are, therefore, comparable with other observers' results. He repeats Yorke and Blacklock's recommendation as to the stripping of the young oil palms and suggests that undergrowth should be cleared and all cleared areas should be planted with Efwatakala grass (*F. minutis*). This recommendation was accepted by the Colonial Government and clearing was commenced, the areas, as cleared, being planted with Efwatakala grass. It may be mentioned at this point that, in the opinion of those competent to judge, this crop has proved a complete success in Sierra Leone. It grows well, spreads rapidly, forms an excellent pasturage for cattle and appears to impede the growth of other vegetation ; the fact that it does not in itself constitute a breeding place for Glossina will be dealt with later.

3. Blacklock (1924) re-examined the same district during August and September of that year, at which time a small area of about 800 square yards had been cleared. He noted a most remarkable reduction in the number of fly caught in the cleared as compared with the uncleared area ; this is shown by his statement that during a total period of ten hours spent in each area, no flies were caught by two boys in the completely cleared area, in distinction to twenty-four and fifty-nine flies caught, respectively, in the partially cleared and uncleared areas. He explains this remarkable reduction by (1) the destruction of Glossina breeding places, and (2) the actual killing of pupae by the trampling of labourers engaged in clearing the bush. This latter suggestion may have some bearing on our observation that fly have since appeared in small numbers in this area after the completion of clearing. Blacklock carefully examined the cleared area for breeding places without success, but, owing to the limited time at his disposal, did not re-examine for pupae the uncleared areas which, during the dry season, had on the previous occasion yielded such a large number. It is possible that if he had examined the uncleared areas, a reduction might also have been found, for, as we show later, *G. palpalis* does not readily larviposit anywhere on the Peninsula during the rains.

From the time of Blacklock's report in 1924, till the commencement of our own observations in 1928, no further investigations were carried out but clearing was steadily continued. Unfortunately,

owing to financial and other considerations, the progress was slow ; however, by the end of 1927 the whole of the western portion of the peninsula had been cleared, i.e., area No. I on the map.   At this time, early in 1928, we found several cases of *T. gambiense* infection among natives in the district and it was decided to re-investigate the question of trypanosomiasis in man and domestic stock on the Peninsula.   It is with this work, carried out in 1928 and 1929, that our present paper deals.   The 1928 part of the investigation was carried out by one of the writers (R. M. G.) and Dr. R. Aidin.   Some of their results have been published in the Sierra Leone Annual Medical and Sanitary Report for 1928.   For the purpose of the present paper this and the 1929 investigation will be considered together.   The main object of the inquiry was to investigate the following points :—

1.   To what extent were the human population and domestic stock infected with trypanosomes, and what were the types of infections.

2.   What proportion of the fly were infected with trypanosomes.

3.   What species of trypanosomes were responsible for the fly infections.

4.   From what source were the fly obtaining these infections.

5.   To what extent had the area so far cleared resulted in a local or general fly reduction.

6.   Were fly breeding in the cleared area and if so at what season of the year.

We propose to show, in the paper which follows, that the facts obtained from our experiments and investigations have enabled us to supply a definite answer to some of these queries, and in the remaining instances to formulate a probable hypothesis.

II. EXAMINATION OF THE HUMAN POPULATION AND DOMESTIC STOCK FOR TRYPANOSOMIASIS

We may state here that the number of persons and domestic animals examined by us was disappointingly small.   This was entirely due to the reluctance of the inhabitants to submit either themselves or their stock to any examination which included gland or vein-puncture, a difficulty not encountered in less civilised parts of Sierra Leone.   This difficulty has already been referred to by other observers (*vide* Blacklock, 1922).

Cape Sierra Leone
Light House & Rest House

No. I AREA

Man of War Bay.

Isolation Camp.

Path

Path

ABERDEEN HILL.
No II AREA

Path

No II
AREA

LUMLEY BEACH.

ABERDEEN VILLAGE

Pirate Bay.

No III AREA

Mangrove Swamp.

ABERDEEN CREEK.

No. IV AREA

Ferry.

Cockle Bay.

MURRAY TOWN.

S

Scale 6 inches = 1 mile.

Yards 0   100   400   500 Yards.

I AREA completely cleared.
II, III and IV AREAS partially
cleared in patches.
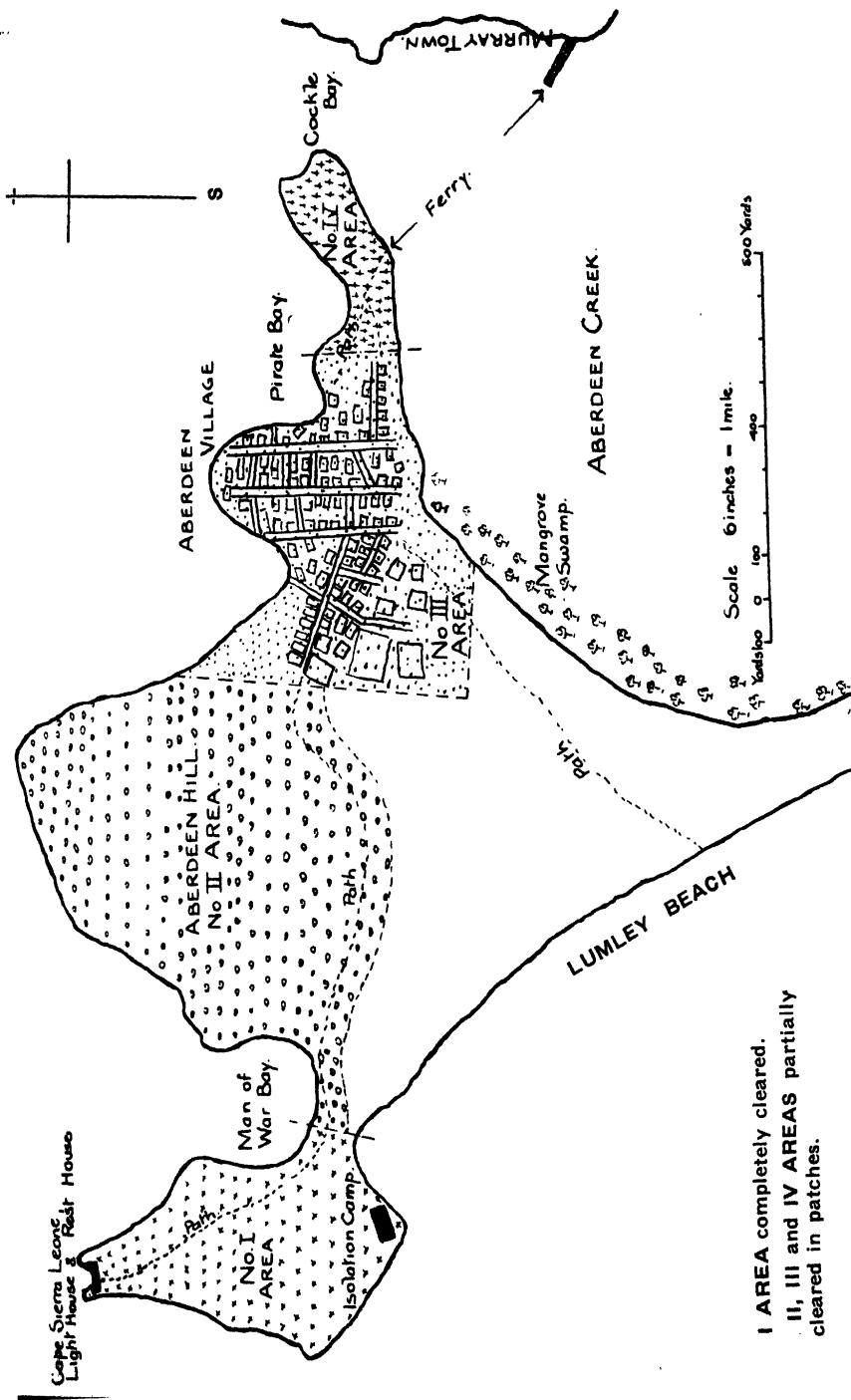
1. *Examination of the human population.* The population of the Cape Lighthouse Peninsula consists entirely of natives and almost exclusively of Creoles. With the exception of a few scattered farms and native squatters' huts, the entire population of the Peninsula is concentrated in the village of Aberdeen which lies at the eastern extremity. The Cape Lighthouse, which formed our headquarters during the investigations and those of the observers already quoted, lies at the extreme western extremity, and houses a native population of about thirty persons, including women and children. The 1921 Colonial census estimates the population of Aberdeen at 500 persons, but the present population, exclusive of the scattered houses already referred to, is probably considerably in excess of this figure.

Our examination was divided into two periods. From June 6th to July 19th, 1928, clinics were held twice a week in the village of Aberdeen. All children attending school were examined, and all adults, whether or not requiring medical attention, were invited to attend. Apart from routine clinical examinations, the following data were recorded : (*a*) location of enlarged lymphatic glands, (*b*) presence of any obvious causes for the enlargement, (*c*) whether such glands were puncturable, (*d*) the results of blood examination, which included a 'fresh film' and stained thick and thin films. From the 23rd July to 3rd August, 1928, and from 30th August to 30th September, 1929, we lived in the rest-house at the Cape Lighthouse Peninsula, and during these periods an attempt was made to re-examine all those cases which had previously aroused suspicions of trypanosomiasis ; gland puncture was performed on as many of these persons as permitted the examination.

The results of these combined examinations were as follows :— two hundred and sixty persons were examined, and of these, 71 were excluded as either being under five years of age, or not resident on the Cape Peninsula. The remaining 189 persons were found to consist of 73 adults (30 males, 43 females) and 116 children (five to fifteen years of age). In each of the 189 cases the peripheral blood was examined by means of (*a*) a 'fresh film,' (*b*) a stained thin film, and (*c*) a · stained thick film. In nineteen of these cases gland puncture was performed. In not a single instance were trypanosomes demonstrated in either

the peripheral blood or the cervical gland juice. The result of this examination of the inhabitants of the Cape Peninsula for trypanosomes was, therefore, completely negative. On the other hand, prior to the commencement of the investigation, we had demonstrated trypanosomes in the blood or gland juice of two individuals living in the village of Aberdeen. As only 189 persons, aged five years and upwards, presented themselves for examination it seems probable that, had it been possible to examine a larger proportion of the population, more cases would have been detected. Finally, certain cases, regarded by us as highly suspicious, were probably true cases of trypanosomiasis in whom partial immunity had developed, and the trypanosomes had become too scanty for detection by the ordinary methods of examination. It is impossible to discuss these cases in individual detail ; it is sufficient to say that certain cases of glandular enlargement accompanied by complaints of frontal headache and lethargy and, in a certain proportion of the cases, by auto-agglutination, appeared to be highly suggestive of trypanosomiasis. It is unfortunate that we were unable in the majority of such cases to carry out gland puncture. That trypanosomiasis may be widespread in a community without trypanosomes being demonstrable in the peripheral blood of the patients, is shown by Macfie and Gallagher (1914) at Eket in Nigeria, where they examined 962 natives and found 222 of these infected with trypanosomes. Amongst this large number of positive cases in not a single instance were trypanosomes demonstrated in the peripheral blood ; in each case they were found in the cervical glands, excision of the gland being resorted to when puncture failed.

Our examination of the population at Aberdeen shows that 24 per cent. of adults and 71 per cent. of children have enlarged cervical glands. This high proportion of glandular enlargement is comparable with Macfie and Gallagher's figures for highly infected districts in Nigeria. These figures are compared together in Table I, together with Gordon's (1927) figures for a similar examination carried out in a village in Sierra Leone where trypanosomiasis has existed in the past.

We have already referred to the fact that certain cases of glandular enlargement were associated with auto-agglutination. The technique used by us consisted in examining a drop of fresh peripheral blood under a coverslip, taking care that both slide and coverslip were

thoroughly clean, This technique is liable to certain errors, but it has the advantage of rapidity and this was necessary in order to examine all the natives awaiting attention at the clinic. Amongst seventy-three adults thus examined, nine (12 per cent.) gave a positive

TABLE I

Showing the incidence of enlarged cervical glands amongst children and adults. (1) Adapted from Macfie and Gallagher's (1914) figures for Mpok, Nigeria, where trypanosomiasis existed in the past but is now almost unknown ; (2) Macfie and Gallagher's figures for Ikot Offong, where trypanosomiasis was very prevalent at the time of their observations ; (3) At Mabang, where trypanosomiasis has occurred in the past but has not been recorded for several years ; (4) At the Cape Lighthouse Peninsula.

	Macfie and Gallagher (1914), Mpok, Nigeria. Total adults, 528. Total children, 297.	Macfie and Gallagher (1914), Ikot Offong. Total adults, 68. Total children, 69.	Gordon (1927), Mabang. Total adults, 129. Total children, 83.	Gordon and Davey (1928-29). Total adults, 73. Total children, 116.
Percentage of adults with enlarged cervical glands ++.	0·4	0	1·5	2·7
Percentage of adults with enlarged cervical glands +.	65·7	35·3	6·9	19·2
Percentage of adults with no enlargement of the cervical glands.	33·9	64·7	91·6	78·1
Percentage of children with enlarged cervical glands ++.	2·7	21·8	11·1	20·7
Percentage of children with enlarged cervical glands +.	86·5	69·5	19·2	47·4
Percentage of children with no enlargement of the cervical glands.	10·8	8·7	69·7	31·9

reaction and among 116 children,. fourteen (12 per cent.) were similarly positive. Four of these cases had their blood re-examined on a later date and all four gave a similar result. There appears to be a certain association between positive auto-agglutination tests and

glandular enlargement; thus, of the twenty-three cases that gave a positive auto-agglutination, sixteen (70 per cent.) were associated with enlarged cervical glands.

(2) *Examination of domestic stock.* The domestic animals on the Peninsula consist of cattle, sheep, goats and dogs, the keeping of pigs being illegal. During the period of our investigation the only cattle resident on the area were two oxen, ' pensioners ' from the Sanitary Department, which had been in residence on the cleared area for five years. These two animals were repeatedly examined by means of ' fresh films,' and finally 10 c.c. of blood were obtained from each by vein-puncture and the centrifuged deposit examined with negative results. Three other oxen were kept for a short time at a small farm which adjoins the Lumley beach, and these also proved to be negative for trypanosomes. On some occasions quite large herds, forty or more animals, can be seen grazing on the grass adjoining Lumley beach. These animals are on their way down from French Africa; during the period that we had the area under observation, herds were only seen on two occasions and then only during the course of one day.

Sheep and goats are numerous but it is impossible to give any exact figure for their numbers, probably 150-200 being kept by the inhabitants. Forty-four sheep and twenty-seven goats were examined by a single ' fresh film,' in one of these animals 10 c.c. of blood being also centrifuged and examined : in no instance were trypanosomes discovered. It is impossible to obtain any exact data regarding the mortality amongst the domestic sheep and goats at various ages, but the result of our enquiries has convinced us that the death rate is low and that the Cape Peninsula forms an excellent breeding place for the local race of sheep and goats, although it seems highly improbable that imported animals, under the present conditions, would survive exposure to infection. This is borne out by the experiments detailed in Table IV, which are referred to later, where it is shown that two out of three imported lambs developed a virulent type of *T. vivax* infection which resulted in their death within a month.

Dogs are very common in the village of Aberdeen, but it was not considered practicable to carry out an examination of their blood; routine laboratory examination of dogs, kept by Europeans in a non-

infected area but occasionally exposed to tsetse-fly when their owners are bathing at Lumley beach, shows that a high proportion of such animals develops *T. congolense* infection.

III. PROPORTION OF FLY INFECTED WITH TRYPANOSOMES

All the flies recorded below in Table II were captured by trained fly boys on the Cape Peninsula, the majority being taken in or around the village of Aberdeen. Flies thus captured were examined as to their species, and all, as in previous surveys, found to be *G. palpalis*. They were then either immediately dissected, or else transferred to clean ' feeding bottles ' and, after twenty-four to forty-eight hours' starvation, fed on experimental animals which had been proved free of trypanosome infection ; after the completion of their feed they were again starved for twenty-four to forty-eight hours and then dissected.

TABLE II

Showing the percentage of infected fly noted by different observers, during various periods, on the Cape Lighthouse Peninsula.

Authority and date	Period of year	Number of flies dissected	Total flies found infected	Percentage of flies found infected
Yorke and Blacklock (1915) ...	December to February	400	21	5·2
Blacklock (1922)	July to August	471	28	5·9
Gordon and Aidin (1928) ...	July to August	209	40	19·1
Gordon and Davey (1929) ...	September	986	96	9·7

These figures show a considerable fluctuation in the proportion of the infected fly in different years, but there would appear to be a definite increase in the percentage of trypanosome infection during the more recent years.

V. THE SPECIES OF TRYPANOSOMES OCCURRING IN INFECTED FLY

We have already quoted Yorke and Blacklock's (1915) figures, showing the anatomical sites of infection with trypanosomes amongst the 400 fly they dissected at the Cape Lighthouse Peninsula.. Basing their opinion on the anatomical sites of infection in the twenty-one infected flies they concluded that of the 400 flies examined, fifteen (3·75 per cent.) were infected with *T. vivax*, four (1 per cent.) with *T. congolense* and two (0·5 per cent.) with a trypanosome belonging either to the *T. congolense* or *T. gambiense* group. They found no salivary gland infection amongst the 400 fly dissected. Blacklock (1922), amongst 471 flies dissected, found one infected in the salivary glands. As he did not include the gut in all his dissections, he draws no conclusion as to the respective proportion of *T. vivax* and *T. congolense* infections.

The present writers during 1928 dissected 209 flies, of which thirty-five (16·7 per cent.) were infected in the proboscis alone, two (1 per cent.) in the proboscis and gut, and three (1·4 per cent.) in the gut alone. In 1929 we examined 986 flies, of which 8·3 per cent. were infected in the proboscis alone, 1·1 per cent. in the gut and proboscis, and 0·3 per cent. in the gut alone. On neither occasion was any gland infection recorded. We summarise these figures below in Table III.

It will be seen from Table III that the great majority of the fly infections are of the proboscis type, and that this is also responsible for the marked increase in the percentage of infected flies occurring in the years 1928 and, to a lesser extent, in 1929. It also shows that flies with a combined gut and proboscis infection were comparatively rare and that no noticeable increase in their numbers occurred in the later surveys.

These anatomical sites of infection suggested that *T. vivax* was responsible for the majority of the infections, and *T. congolense* for the remainder. During our 1929 survey we determined to try and establish with certainty the exact species of trypanosome responsible, by means of careful morphological examinations of stained preparations from infected flies and also by attempting to transmit the different types of infections to sheep and dogs.

TABLE III

Showing the percentage of infections occurring in the proboscis alone, proboscis and gut, and gut alone, amongst the flies dissected by different observers during various periods on the Cape Lighthouse Peninsula.

Authority and date	Total flies examined	Percentage of flies found infected (other than salivary gland infections)	Percentage of flies with proboscis infection alone, i.e., *T. vivax* type	Percentage of flies with proboscis and gut infections i.e., *T. congolense* type	Percentage of flies with gut infection alone, i.e., gambiense-congolense type
Yorke and Blacklock (1915b)	400	5·25	3·75	1·0	0·5
Blacklock (1922) ...	471	5·7*	...	...	...
Gordon and Aidin (1928)	209	19·1	16·7	1·0	1·4
Gordon and Davey (1929)	986	9·7	8·3	1·1	0·3

* Gut infections omitted.

(1) *Experiments in the transmission of fly infections to sheep and dogs.* Four lambs and three puppies were used in the experiments. In the case of the lambs, before feeding experiments were commenced, 10 c.c. of their blood were centrifuged, and in the case of the puppies, numerous fresh preparations were examined ; all were found to be negative. The animals were examined by means of ' fresh films ' for various periods after fly had been allowed to feed on them. Nine hundred and twenty-two fly were fed on the animals and subsequently dissected. The results of the experiments are shown in Table IV.

The large number of flies used in the puppy experiments was necessitated by the unexpected scarcity of gut-infected flies which happened to occur at the time that these experiments were in progress.

(2) *Morphological appearances.* Lloyd and Johnson (1924) state that *T. vivax* infections in the fly may be sharply differentiated from *T. congolense* by the fact that *T. vivax* obtained from the hypopharynx always possesses a free flagellum, whereas, in *T. congolense* infections of the same site the flagellum is invariably absent. Our

examination of ninety-six infected fly is in agreement with their statement, for in no case where a proboscis infection was accompanied by a gut infection did we find trypanosomes with a free flagellum in the hypopharynx. The entire absence of salivary gland infections precludes the possibility of the presence of *T. gambiense* or *T. brucei* amongst the flies dissected.

TABLE IV

Showing the results of feeding 922 *G. palpalis*, caught on the Cape Lighthouse Peninsula, on lambs and puppies.

No.	Animal	From where obtained	Total fly fed	No. of flies with proboscis infection	No. of flies with proboscis and gut infections	Results of feeding infected fly
1	Lamb	Tsetse free area	40	3	2	Developed *T. vivax* and died 25 days later.
2	Lamb	Tsetse free area	64	3	2	Developed *T. vivax* and died 35 days later.
3	Lamb	Tsetse free area	35	2	1	Developed no infection during 35 days' observation, then injected with infected blood from lamb No. 1, developed *T. vivax* 11 days later.
4	Lamb	Tsetse Infected area	39	3	0	Developed no infection for 21 days subsequent to the last infective fly feed.
5	Puppy	Tsetse free area	136	14	0	Developed no infection. (Observed 1 month).
6	Puppy	Tsetse free area	374	36	4	Developed *T. congolense* infection.
7	Puppy	Tsetse infected area	234	15	0	Developed no infection. (Observed 1 month).

These transmission experiments and the morphological examinations of trypanosomes found in infected flies, show clearly that the species of trypanosomes occurring in *G. palpalis* on the Cape Lighthouse Peninsula are *T. vivax* and *T. congolense*. The small percentage of flies infected in the gut alone might be carrying a *T. grayi* infection, but the absence of crocodiles renders this very unlikely.

V. THE SOURCE OF *T. VIVAX* AND *T. CONGOLENSE* INFECTIONS

We are in agreement with previous observers' statements that the larger forms of wild life, with the exception of monkeys, squirrels, iguanas, etc., appear to be comparatively rare on the Peninsula, but deer undoubtedly exist in small numbers, and during 1929, one Harness Antelope and its kid were living in the cleared area.   By means of a game-drive, consisting of about 200 beaters, an attempt was made to drive wild life from the Peninsula through the narrow isthmus at Man-o'-War Bay but, owing to the denseness of the bush, the drive was not successful and only one deer was seen.   No attempt was made to examine the blood of wild animals, as their rarity in comparison with the relatively large numbers of sheep, goats, and dogs, renders it unlikely that they form any important source of infection. These latter hosts must now be considered.   The small proportion of *T. congolense* infections are almost certainly derived from dogs.   Our failure to infect sheep by feeding *T. congolense* infected flies upon them is of interest and suggests that they do not form a reservoir in this district.* Goats may be a source of *T. vivax* and *T. congolense* infection, but we carried out no transmission experiments.

In order to account for the very high proportion of flies infected with *T. vivax*, shown especially in the 1928 investigation, it is obvious that there must exist on the Peninsula a large number of animals capable of carrying on the infection in the fly.   It would at first sight appear that sheep and goats are unlikely to be the cause, as judged by our failure to discover trypanosomes in the peripheral blood of the sixty-seven adult animals examined by means of a single ' fresh film.'   This apparent absence of trypanosomes from the peripheral blood of sheep and goats is, however, probably accounted for by these animals becoming infected when very young and developing a partial immunity.   Such an immunity would reduce the number of trypanosomes appearing in their peripheral blood to so low a figure that a single ' fresh film ' preparation would fail to reveal trypanosomes in this, or subsequent infections.   In one instance, namely Lamb No. 1 referred to in Table IV, we have observed this

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* After the completion of our experiments in Sierra Leone, sheep No. 3 (referred to in Table IV) was shipped to England where it subsequently developed *T. congolense*, in addition to its existing *T. vivax* infection.

phenomenon. This animal developed a *T. vivax* infection as a result of fly being fed on it. The disease rapidly developed so that four or five trypanosomes were easily found in a single coverslip preparation. For some weeks after this, however, the concentration of the trypanosomes diminished until even the examination of several ' fresh films ' failed to reveal their presence, but at this time a coverslip preparation from a centrifuged specimen of 10 c.c. of venous blood, showed a large number of trypanosomes. It is obvious that such an animal, although apparently negative when examined by means of a few ' fresh films,' would still be capable of infecting a high proportion of the Glossina which fed upon it. Pomeroy and Robinson (1929) have observed a similar state of affairs in bullocks infected with *T. vivax* in the Gold Coast.

VI. THE RESULTS OF BUSH CLEARING ON REDUCING THE NUMBER OF FLY

Clearing of the Cape Lighthouse Peninsula was not commenced until 1923, from which date till the present time it has been continued, whenever labour and funds were available. The main clearing has been from the extreme western portion of the Peninsula towards the village of Aberdeen, situated at the eastern extremity, but certain disconnected clearings of intensely infested areas near the village have also been made. The attached map shows the extent of the main clearing which has so far been accomplished. From this it will be seen that the entire western extremity and the isthmus connecting it to the mainland have been cleared. The observations which follow are mainly concerned with determining whether this complete clearing has resulted in a reduction in the proportion of fly captured on the cleared area, as compared with the proportion of fly captured on the uncleared and partially cleared areas, constituting the remaining portion of the Peninsula. In addition we investigated whether any general reduction in the total number of fly throughout the Peninsula had resulted from this isolated clearing. It is obvious that such observations should be made at the same season in various years, and our examinations, carried our during 1928 and 1929, were therefore undertaken in July, August and September, in order to coincide, so far as possible, with the periods of survey of previous workers.

A comparison of these surveys is simplified by the fact that, as already recorded, *G. palpalis* is the only species of tsetse occurring in the district.

Yorke and Blacklock (1915a) stated that the fly were common throughout the Peninsula, but did not express this numerically. In Table V, we show the results obtained by ourselves in 1928 and 1929 and compare these figures with those of Blacklock, in 1922, before the clearing had commenced, and in 1924, some time after its commencement.

TABLE V

Showing the number of flies captured per boy per hour, as noted by different observers during various periods, on the Cape Lighthouse Peninsula.

Authority and date	Uncleared area		Cleared area		Flies per boy per hour captured in uncleared area	Flies per boy per hour captured in cleared area
	Total time spent in hours	Total fly captured	Total time spent in hours	Total fly captured		
Blacklock (1922). Before clearing commenced ...	57	272	...	...	4·8	
Blacklock (1924). A few months after clearing commenced	20	83	72	0	4·2	0
Gordon and Aidin (1928) ...	123	236	30	62	1·9	2·1
Gordon and Davey (1929)...	283	745	290	50	2·6	0·17

This table would appear to prove that clearing has reduced the number of fly occurring in the cleared area as compared with the uncleared area, and has been associated with a similar though lesser reduction in the number of fly over the whole Peninsula. This observation is supported by the fact that far fewer pupae were collected by us in our 1929 investigation than were found by Yorke and Blacklock, in a similar investigation, which they conducted in 1915, long before any clearing had been commenced. Blacklock's observation of the entire absence of fly from the cleared area in 1924 is an extremely interesting one. That they really were absent

appears to be proved by the fact that he offered a reward of one shilling for every single fly captured on the cleared area and that this reward was never claimed, although the same boys captured numerous fly in the immediately adjacent uncleared area. He believes that not only were fly prevented from larvipositing by the destruction of their breeding places, but that pupae already deposited had been destroyed by the trampling of the clearing gang.

We can offer no explanation of the relatively large proportion of fly captured on the cleared area during 1928.

To sum up the situation at this stage of the investigation ; from the facts already noted, we believe we are in a position to state that whereas human trypanosomiasis is endemic on the Peninsula and that chronic benign cases probably exist undetected, it only plays a negligible part in accounting for the high percentage of infected flies (5 per cent. to 19 per cent. according to various observers). This infection is due for the most part to *T. vivax* and, to a much less extent, to *T. congolense*. The chief source of the *T. vivax* infection is undoubtedly sheep, and possibly goats. Dogs are, in all probability, mainly responsible for the *T. congolense* infection in the remaining small percentage of infected flies. This high percentage of infected flies which apparently obtain their infection from a reservoir of native domestic animals, renders the Cape Lighthouse Peninsula, under such circumstances, worthless as a site for the establishment of an experimental cattle and sheep station. We have also shown that the very small clearing so far effected has resulted in a most remarkable reduction in the number of fly occurring in the cleared, as compared with the uncleared areas. It remains to be shown if a further extension of the clearing will result in reducing the number of fly occurring on this area to a figure of negligible importance. This involves a consideration of the local breeding habits of *G. palpalis* and the effects upon them of bush clearing.

VI. LOCAL BREEDING HABITS OF *G. PALPALIS* AND THE EFFECTS UPON THEM OF BUSH-CLEARING

Yorke and Blacklock (1915a) proved clearly that the breeding places of *G. palpalis* on the Cape Lighthouse Peninsula are almost exclusively confined to the shade of the oil palm (*Eloeis guineensis*), where pupae were to be found in very large numbers. Their observations were confined exclusively to the dry season. A subsequent

observation by Blacklock (1924) of a small portion of this area, which had been recently cleared, failed to reveal any pupae but, for the reasons already given, he did not carry out any control observations in the uncleared area. It is important to note that this investigation, in contradistinction to that of 1915, was carried out during the wet season. During 1928 no observations were made on the breeding habits of *G. palpalis* and the remarks that follow refer exclusively to our work in 1929.

On various occasions during the dry seasons of the past four years, we have confirmed Yorke and Blacklock's observation of 1915 regarding the finding of pupae under the shade of oil palms, although we never succeeded in collecting nearly as many pupae as these authors recorded. During September, 1929 (i.e., during the rains) we examined very thoroughly various likely sites on the cleared area for pupae. The method we employed was as follows. A suitable site, usually a young oil palm which had escaped the notice of the clearing gang and which had its lower petioles intact, was selected, the neighbouring bush cut away, and finally the surrounding earth or sand for a distance of two to three feet was removed to a depth of one inch. This earth was placed in a receptacle, labelled, brought back to the laboratory and subsequently examined by scattering it over white enamel trays. All work was carried out under our personal supervision, and we found it essential to make use of natives, whom we had carefully trained, in order to avoid any unnecessary trampling or removing too deep a layer of earth.

That a considerable quantity of ground suitable for breeding places, was examined in this thorough manner is shown by the fact that it required about four hours to examine the material collected from a single site and that twelve such sites were examined on the cleared area. Our results completely confirmed Blacklock's observation in 1924 : not a single pupa was discovered.

In order to control this result we next proceeded to examine, in an exactly similar manner, ten selected sites on the uncleared area, including several sites which on previous occasions, when examined during the dry season, had yielded pupae. Contrary to our expectations we found not a single pupa in nine of the selected sites and only a solitary pupa case in the remaining one. Assuming that the sites we selected were suitable ones, and previous experience convinced us that such was the case, only two explanations for the absence of

pupae are possible. Either fly are no longer breeding on the Peninsula or else they were not larvipositing up to the time that we made our observations. In view of the large number of fly which we have shown still to be present in this area, and that fly on the neighbouring mainland are far less numerous than on the Peninsula, the first explanation appeared to us to be highly improbable. In order to be sure that fly were still breeding on the uncleared area and also to ascertain to what extent they were breeding on the cleared area, we examined similar sites selected from the same districts of the cleared and uncleared areas during the dry season of 1930 with the following results.

During February, 1930, using an exactly similar technique to that already described, we examined ten likely sites on the cleared area. At this time the clearing was so complete that these ten sites represented, in our opinion, all the suitable breeding places. This examination only resulted in the finding of two pupa cases. In distinction to this an exactly similar examination of twenty sites on the remaining uncleared area yielded thirty-one pupa cases. Of the thirty-three pupa cases, five contained living pupae which eventually hatched in the laboratory.

Thirty-two of the thirty-three pupa cases were found under unstripped young oil palms, although the earth below shrubs, fallen logs and in rock crevices was also examined. The remaining pupa case was found in the earth inside a hollow baobab tree from which Yorke and Blacklock, in 1915, recorded the finding of eighteen puparia.

This restriction of larvipositing to the dry season, on the part of G. *palpalis*, in Sierra Leone, is extremely interesting and is further confirmed by certain other observations made during 1928 and 1929, on the proportion of female fly which contained larvae. During July and August, 1928, and the first half of September, 1929, not a single larva was recorded amongst the fly dissected, but from the second half of September to the close of our observations at the end of October, a more or less steady increase in the proportion of female flies containing larvae was noted. These results are shown in the following graph.

It is of interest to record that third instar larvae were only observed *in utero* during the latter half of October.

In view of these results, we are inclined to think that *G. palpalis* does not breed on the Cape Peninsula during the wet season. We have proved that they certainly do not breed there during the latter months of the wet season, but further observations are required to
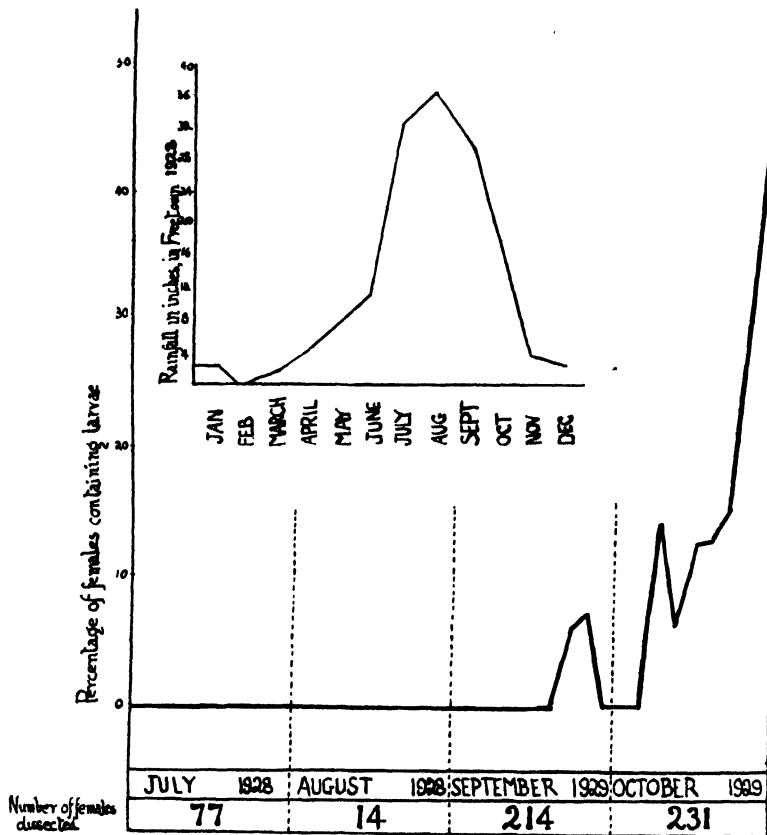
Graph showing the increase in the proportion of pregnant *G. palpalis* towards the conclusion of the wet season, as noted on the Cape Lighthouse Peninsula in 1928 and 1929.

discover if this inhibition extends throughout the wet season, and also to show for how long during the dry season the larviposition continues. Johnson and Lloyd (1923) discussing the breeding habits of *G. palpalis* in Northern Nigeria write as follows :—

' Although this fly is prevalent in many places that we have visited, it was really numerous only on the Niger at the end of the rains, on the large rivers of Nassarawa, and on the Benue about Abinsi. Consequently, searches for pupae revealed only small numbers. They were collected in twenty positions, in every

case in sand, with or without vegetable debris overlying it. These were always very close to water. In fourteen instances there was shrubby undergrowth above the pupae or closely backing the sand-bank in which they were found; twice (eighteen and one pupae respectively) they were below sloping tree-trunks; once (thirteen pupae) focussed around a small upright dead stump with thin shade 20 feet above; and once (two empty cases) very exposed with the scantiest of high shade above them. In eighteen cases there were high, thin ever-green shade, and in two instances this was lacking, there being shrub shade only. The positions are thus quite similar to those described for this species in East Africa. Particularly favourable sites were the high sand-banks thrown up where a small stream joins a larger one. In one such spot 140 pupae were collected by two searchers in a few minutes.

During the rains the searches failed to reveal any pupae beyond an occasional old case, all the normal breeding grounds being then inundated or water-logged. There was also a little evidence obtained by dissections that the rate of breeding is restrained in the height of the rains and at their close. In the late rains (September and October), out of fifty-nine females examined only thirteen (22 per cent.) were pregnant; while in the dry season (December, January, March) and early rains (April), out of 172 females dissected, 117 (68 per cent.) contained larvae.'

It would appear, therefore, that *G. palpalis* larviposits at similar periods of the year in Sierra Leone and Northern Nigeria. The sites of larviposition, however, appear to be different. In Sierra Leone the finding of pupae is almost exclusively confined to the shade of young oil palms sometimes far from water; in Nigeria, on the other hand, the main object of the fly appears to be to deposit its larva near water, and in order to achieve this it will accept almost any available type of shade. Our observations in Sierra Leone clearly invalidate any conclusions, regarding the effect of clearing, based on the absence of *G. palpalis* pupae from suitable breeding sites examined during the wet season. On the other hand, our investigations conducted during the dry season appear to show that far fewer fly are larvipositing in the cleared than in the uncleared areas, and that the local clearing has reduced the number of pupae occurring in both the cleared and uncleared areas at the Cape Light-house Peninsula.

VII. SUMMARY

1. Evidence is produced that human and animal trypanosomiasis have been endemic on the Peninsula at Sierra Leone, at any rate since the beginning of observations in 1915.

In the case of human trypanosomiasis, only a few cases of *T. gambiense* infection of the blood or glands have been recorded

amongst such of the Creole population as permitted this form of examination. We are of the opinion, however, that chronic benign cases are more numerous amongst the population than these findings suggest ; for there was found to exist in this area an abnormally high proportion of the natives, especially children, with enlarged cervical glands. Some of these also exhibited other signs and symptoms such as auto-agglutination, frontal headache, lethargy, etc., very suggestive of trypanosomiasis. It seems likely that some of these were true cases of trypanosomiasis in whom partial immunity had developed and had rendered the number of trypanosomes too scanty for detection except by repeated examinations.

In the case of domestic animals, such as sheep, it has been demonstrated that the local animals must almost certainly become infected with *T. vivax* early in life, and that such animals, at a later stage, although still capable of infecting a high proportion of the *G. palpalis* which feed upon them, do not possess sufficient trypanosomes in the peripheral blood for these to be demonstrable by the ordinary coverslip preparations.

2. It is shown that throughout the investigations from 1915 to 1929, a high proportion (5·2 per cent. to 19·1 per cent.) of the fly were found infected with trypanosomes. It is further shown that a marked increase in the proportion of infected fly occurred during the year 1928 and to a lesser extent during the year 1929.

3. As a result of a series of observations, involving transmission experiments with sheep and dogs and the morphological examination of stained preparations made from infected flies, it is proved that this high proportion of infection amongst the fly is due to *T. vivax* and *T. congolense*, the former representing the great majority of the infections and being mainly responsible for the marked rise in the infection rate observed by us during the years 1928 and 1929.

The *T. gambiense, T. brucei* type of infection is of rare occurrence amongst fly captured on the Peninsula. Thus of approximately 1,200 fly dissected by us, none were found infected in the salivary glands, while of 800 fly dissected previous to our investigations, only a solitary positive gland infection was noted.

4. Transmission experiments conducted with sheep show that these animals are in all probability the main reservoir from which the *G. palpalis* obtain their *T. vivax* infection. Similarly, dogs (and

possibly domestic goats) are responsible for the *T. congolense* type of infection.

Wild game is shown to be of negligible importance as a reservoir of trypanosome infection.

5. The clearing so far accomplished on the Cape Peninsula has resulted in a marked reduction in the number of fly on the cleared area, and has been associated with a similar though lesser reduction on the uncleared area. Thus the average figure for the number of fly captured per boy per hour before clearing (1922-1924) was 4·5, while our figures, recorded after partial clearing, show an average of only 1·1 fly on the cleared area and 2·3 fly on the remaining uncleared area. These figures are supported by records of the number of pupae collected on the cleared and uncleared areas, which show that *G. palpalis* rarely larviposits in the cleared area, while larviposition on the remaining uncleared, or partially cleared, area has been greatly reduced.

6. As a result of a series of observations on the number of pupae found during the wet and dry seasons and on the state of pregnancy of female flies dissected during a part of the period, we are in a position to state that although the selected sites of larviposition appear to be essentially different from those described for Nigeria, yet the breeding habits of *G. palpalis* on the Cape Peninsula, Sierra Leone, are similar in that larviposition is almost exclusively confined to the dry season.

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PLATE VIII

EXPLANATION OF PLATE VIII

Fig. 1. Cape Peninsula, No. II area, showing dense bush extending down to the shore.

Fig 2. Cape Peninsula, No. II area, showing uncleared area.

Fig. 1

Fig. 2
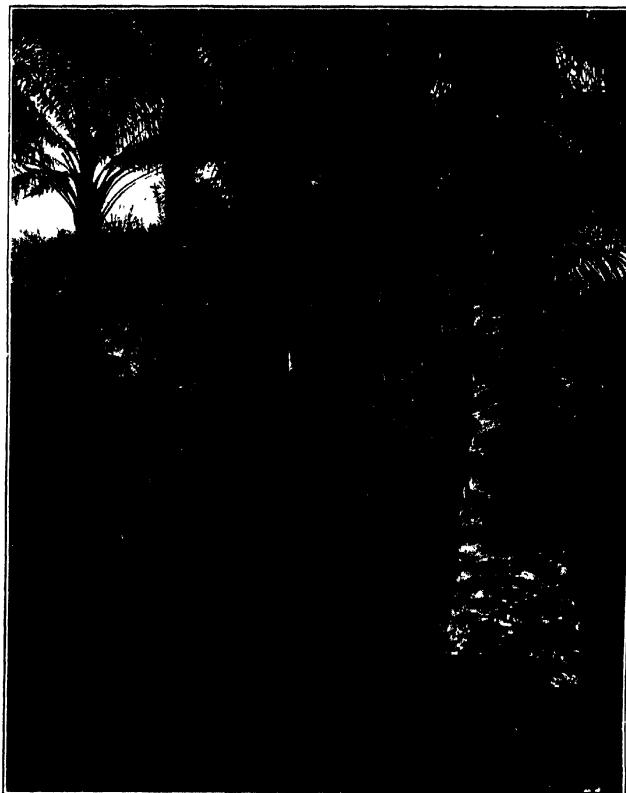
EXPLANATION OF PLATE IX

Fig. 3.   Cape Peninsula, No. II area, showing partially cleared
portion.

Fig. 4.   Cape Peninsula, No. I area, showing park-like appearance
after clearing and planting with *F. minutis*.

FIG. 3

FIG. 4

EXPLANATION OF PLATE X

Fig. 5.   Cape Peninsula, No. IV area, showing clearing near mangrove
          swamps.

Fig. 6.   Cape Peninsula, No. III area, showing outskirts of Aberdeen
          village.

FIG. 5

FIG. 6

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SMITH, J. (1900). Enlargement of the spleen in malaria. *Jl. of Pathometry*, **1**, 1-20.

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THE GLYCOLYTIC POWER OF TRYPANOSOMES *(TRYPANOSOME EVANSI)* IN VITRO

BY

A. GEIGER

I. J. KLIGLER

AND

R. COMAROFF

(*Department of Hygiene and Bacteriology, Hebrew University, Jerusalem*)

The nature of the metabolism of trypanosomes and its effect on the infected host is still a subject of controversy. In a recent publication (1929) it was pointed out that there were two opposing theories. One group of investigators maintains that the pathological changes are due to a toxin elaborated by the trypanosomes, while the other attributes the results to a depletion of the glucose and glycogen reserves.

In our investigation (1929) we could not confirm the toxin theory. We found, however, that the blood of trypanosome infected rats contains an abnormal concentration of lactic acid, which increases progressively with the development of the infection. These findings led to the assumption that the abnormal concentration of lactic acid in the blood of trypanosome infected rats was a consequence of the sugar fermentation by the infecting organisms, and that the progressive production and accumulation of lactic acid led to a progressive depletion of the alkali reserve, the process ultimately resulting in death from acidosis.

The above assumption presumes an active production of lactic acid by the trypanosomes. We have attempted to test the validity of this hypothesis by ascertaining quantitatively the rate of glucose utilization and of lactic acid production by trypanosomes, under controlled conditions, *in vitro*. Owing to the inability to cultivate these organisms, the experiments were necessarily of short duration. The results were, however, checked in a variety of ways under

different conditions, and there can, therefore, be no doubt that the data presented below indicate correctly the glycolytic properties of the trypanosome studied (*T. evansi*).

METHODS

The procedure consisted in following the activity of the organisms in media of given composition under prescribed conditions. A given quantity of infected blood was added to a Locke solution adjusted to a given pH, and containing a given concentration of glucose. The number of trypanosomes and the concentration of lactic acid were determined at the start of the experiment and at various short intervals thereafter. In a number of experiments, the glucose concentration was also determined simultaneously. In this manner the progress of events was followed and an approximate idea obtained of the rate of sugar destruction and acid production by a unit quantity of trypanosomes.

The trypanosomes were counted in a blood counting chamber, thus obtaining absolute numbers. In order to assure uniform distribution, the mixtures were always shaken thoroughly before the count was made. Only motile trypanosomes were counted.

The lactic acid was determined in two ways. In some experiments the direct method of Friedmann, Cotonio and Schaffer (1927) was used. In others the indirect method of CO_2 liberation from a bicarbonate solution was employed. In a number of experiments both methods were used simultaneously with corresponding results.

The amount of lactic acid produced by a unit number of trypanosomes (1 billion) per c.c. of medium in the course of one hour was computed by the following equation :*

$$\text{Lactic acid per billion trypanosomes per hour} = 10,000 \frac{\text{Mgm. lactic acid produced in 100 c.c. solution.}}{\text{No. of tryps. per c.mm.} \times \text{time in hours.}}$$

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* The following example will illustrate the method of computation.

In an experiment lasting 2¼ hours, an average number of 30,000 trypanosomes per c.mm. produced 24 mgm. of lactic acid per 100 c.c. solution. From this it follows that the amount of lactic acid per 100 c.c. produced by 1,000,000,000 organisms per hour may be computed from the following equation :—
$$3 \text{ billion} : 24 = 1 \text{ billion} : x,$$

$$\text{or } x = \frac{24}{3} = \text{amount of acid produced in 2¼ hours.}$$

$$\text{In one hour } x = \frac{24}{3 \times 2\frac{1}{4}} = 3\cdot2 \text{ mgm.} = \frac{1 \text{ billion tryps.} \times \text{mgm. lactic acid produced in 100 c.c.}}{\text{Number of tryps per 100 c.c.} \times \text{duration of experiment.}}$$

$$\text{Or more simply} = \frac{10,000 \times \text{mgm. lactic acid per 100 c.c.}}{\text{Number of tryps. per c.mm.} \times \text{time in hours.}}$$

In view of the fact that the direct method of lactic acid determination is not always accurate where metabolic products of bacteria and protozoa are concerned, the indirect method of lactic acid determination has been used as a check. A similar method has been used by Warburg (1924). It is based on the fact that when a given quantity of acid is added to a bicarbonate solution an equivalent amount of CO_2 is evolved. Consequently, if the CO_2 content of the bicarbonate solution is measured before and after the acid is produced, the increase in the acid can be calculated from the CO_2 liberated. Since the acid production by the trypanosomes is a continuous one, it is important that the solution be kept in equilibrium with a relatively large gas space by constant shaking.

The following is a brief summary of the technique employed :—

A mixture was prepared consisting of a Locke solution, containing known amounts of bicarbonate and glucose ; in a number of experiments a given quantity of normal guinea-pig serum was also added. To these solutions a measured quantity of blood from a trypanosome infected rat was added. The trypanosome containing mixture was divided among a number of Barcroft saturators to which CO_2 gas was added to give the desired partial pressure. The gas pressure was adjusted according to the manometric method described by Van Slyke ; the oxygen tension was always atmospheric and a requisite quantity of air was replaced by CO_2 and the required amount of oxygen. The total gas pressure was maintained at about 10 mm. Hg. greater than atmospheric in order to facilitate the withdrawal of test samples.

The pH was regulated by the CO_2 concentration. It was computed from the CO_2 in the solution and the CO_2 tension in the gas mixture. According to Hasselbach (1916), the pH $= pK + \log s$ $+ \log \dfrac{NaHCO_3}{H_2CO_3}$, where s = degree of dissociation of the bicarbonate.

The determination of glucose in our solutions presented serious difficulties, which we shall have occasion to discuss more fully below.

The first sample was withdrawn after the saturators had been agitated at least ten minutes. In this sample a trypanosome count was made, the CO_2 content determined by the manometric method of Van Slyke (1927), and the glucose concentration by the Hagedorn-Jensen (1923) method. The saturators were then placed in incubators

and constantly agitated. At given intervals, samples were removed as above, and analysed. The lactic acid produced and glucose consumed by a given number of trypanosomes during a given time interval was determined from the differences between the first and subsequent samples.

Lactic Acid Production.

A Locke solution was used containing 0·4 per cent. glucose. To this, defibrinated blood from a heavily infected rat was added in the ratio of 1 : 20. The mixture was divided into several saturators containing various CO_2 tensions. In order to eliminate the intensive autoglycolysis of the blood which usually occurs during the first half hour, the first test was made half an hour after the experiment was set up. A sample was withdrawn in the absence of air into a pipette provided with a glass stopcock, and immediately transferred to a Vay Slyke apparatus to determine the CO_2 content. At the same time a drop was taken for a trypanosome count. The process was repeated after given intervals.

Autoglycolysis of the Blood Cells.

As is well known, blood cells of freshly drawn blood have a considerable glycolytic power. In a number of control experiments it was found that during the first half-hour, normal rat blood will produce a 4·4 mgm. lactic acid per 100 c.c. of blood. This activity is reduced during the next three to four hours, so that the average amount of lactic acid produced per hour is only 1·7 mgm. per 100 c.c. Since in our experiments highly diluted blood was used, and the amount of lactic acid produced by the trypanosomes was relatively much greater, the small amount of lactic acid due to autoglycolysis after the first half-hour is negligible, and was therefore disregarded in the calculations.

RESULTS

The data obtained in the first series of experiments are presented in Table I. It is apparent that there is active consumption of glucose associated with the production of lactic acid. It is also apparent that the number of trypanosomes usually present in heavily infected rats long before death sets in, produces an amount

TABLE I. Lactic acid production by *Trypanosome evansi*.

Date	Temp. °C.	CO₂ tension mm. Hg.	Average No. of tryps. per c.mm.	Duration of experiment (in minutes)	Millimols CO_2 per litre at the start	Millimols CO_2 per litre at the end	Millimols CO_2 per litre evolved	Average pH	Lactic acid produced mgm. %	Mgm. lactic acid produced by 1 billion of tryps. per hour
13.6.29	24	80	141,000	70	8,876	5,855	3,021	6·41	27·19	1·58
13.6.29	24	80	85,000	105	8,876	6,967	1,909	6·45	17·18	1·28
16.6.29	24	40	61,000	60	10,539	8,665	1,874	7·01	16·87	2·76
16.6.29	24	40	73,500	85	10,539	7,377	3,162	6·97	28·46	2·73
16.6.29	24	80	36,000	145	10,539	7,963	2,576	6·57	23·18	2·50
17.6.29	24	80	32,500	105	9,086	7,552	1,534	6·50	13·81	2·46
20.6.29	24	20	59,500	60	6,089	4,449	1,640	7·06	14·76	2·48
24.6.29	24	80	26,000	150	9,017	6,960	2,057	6·46	18·51	2·66
24.6.29	24	20	30,500	150	9,017	6,345	2,672	7·25	24·05	3·37
4.7.29	24	80	45,000	240	15,815	9,436	6,379	6·77	57·41	3·18
4.7.29	24	20	45,000	240	12,555	2,806	9,749	7·25	87·74	4·87
5.7.29	24	94	52,000	240	16,134	8,294	7,840	6·65	70·56	3·39
5.7.29	24	20	52,500	300	13,971	1,988	11,983	7·27	107·85	4·11
11.9.29	24	40	21,000	60	14,860	13,850	1,010	7·22	9·00	4·3
24.9.29	24	80	23,600	155	22,490	19,620	2,180	7·06	19·62	3·3
24.9.29	24	30	25,000	90	15,750	13,890	1,860	7·31	16·70	4·4

NOTE.—The determinations were made in mixtures of one part blood and two parts Locke solution. The concentration of glucose was 0·4 per cent.

of lactic acid which can readily lead to an acidosis. This is evident from a simple computation. A rat of 120 grammes would have about 10 c.c. of blood. The amount of bicarbonate in the blood is about 15 millimol per litre ; of phosphate, 2 millimol. The 10 c.c. of blood contain, therefore, 0·17 millimol of available alkali. As can be seen on Table I (p. 323), 1 billion of trypanosomes per c.c. at pH 7·2-7·4 produce on an average 4·0 mgm. of lactic acid per hour. This is equivalent to about two and a half times the alkali content of the blood. Part of this lactic acid is undoubtedly oxydised and eliminated ; part is neutralized by the alkali reserve of the tissues, but, as was shown in our previous communication (1929), an excess remains which ultimately results in a severe progressive acidosis.

Consumption of Glucose.

Parallel with the determination of the lactic acid production, we also measured the consumption of glucose. As indicated above the Hagedorn-Jensen method was used for these determinations.

Table II shows in parallel columns the glucose consumed and lactic acid produced by 1 billion organisms per hour. It will be noted from the table that the production of 3-4 grammes of lactic acid corresponds to a consumption of about 10 grammes of glucose.

TABLE II

Glucose consumption and lactic acid production by Trypanosomes.

Date of experiment	Glucose consumed per hour by 1 billion tryps. (in mgm.)	Lactic acid produced per hour by 1 billion tryps. (in mgm.)
13.5.29	12·5	4·7
15.5.29	7·5	1·6
19.5.29	6·7	1·4
22.5.29	3·0	1·1
22.5.29	10·0	3·6
19.6.29	13·0	4·2
21.6.29	11·6	3·9

It is difficult to say to what extent this relation is a correct representation of the facts. It is known that micro-organisms produce reducing substances of unknown character. This is probably also true of trypanosomes. It is conceivable, therefore, that more sugar has actually been consumed and that the sugar determinations represent these reducing substances as well as the remaining glucose.

That this is probably the case is indicated by the fact that when media with small concentrations of glucose were used, the production of lactic acid was associated with an increase in the glucose content. This peculiar phenomenon is illustrated by the results of the following experiments :—

EXPERIMENT 1. A mixture consisting of 25 c.c. Locke solution and 0·75 c.c. infected blood was divided into saturators as above. From one of the apparatus the O₂ was exhausted and replaced with N. The glucose content was determined at the beginning and end of the experiment with the following results :

	Glucose content mgm. per cent.		No. of tryps. per cm.		Duration of experiment
	Beginning	End	Beginning	End	
With O₂	0·099	0·121	38,000	6,000	85 minutes
Without O₂	0·095	0·131	38,000	29,000	60 „

EXPERIMENT 2.—A mixture containing 25 c.c. Locke solution and 0·6 c.c. infected blood.

	Glucose content mgm. per cent.		No. of tryps. per cm.		Duration of experiment
	Beginning	End	Beginning	End	
With O₂	0·075	0·075	30,000	9,000	2 hours
Without O₂	0·075	0·089	30,000	28,000	2 „

Similar results were obtained in a number of experiments in which the sugar content of the medium was less than 0·1 grammes per cent. When larger concentrations of glucose were used, a decrease invariably resulted, but because of the results obtained

when small amounts of glucose were used, it is not possible to say to what extent the decrease is a correct measure of the amount of glucose consumed. If we assume that 2 mols. of lactic acid are produced from 1 mol. of glucose, then it is apparent that the amount of glucose consumed must have been greater than indicated by the data given above (Table I).

DISCUSSION

The experiments reported in this communciation leave little room for doubt that *T. evansi* consume glucose *in vitro* with great avidity* with the production of lactic acid. There is no reason to doubt that the same process occurs also *in vivo*, although very likely with modifying circumstances. While *in vitro* the glucose is rapidly used up and the accumulated lactic acid quickly leads to a state which inhibits the development and destroys the trypanosomes ; *in vivo* the glucose is rapidly replenished and the lactic acid is partly oxidised and partly eliminated after neutralization. It is apparent, however, that as the infection progresses the oxidation processes are depressed, and the neutralization of large amounts of lactic acid leads to a depletion of the alkali reserve and an acidosis.

It is conceivable that a similar process goes on in malaria infections. The strikingly more favourable results obtained in treatment of malaria with the administration of bicarbonates (Sinton, 1926) is highly suggestive. At any rate, the rôle of active glycolysis with the production of lactic acid in the pathology of trypanosome and malaria infections deserves serious consideratiôn.

CONCLUSIONS

T. evansi consumes dextrose *in vitro* with great avidity with the production of lactic acid.

It is suggested that this metabolic characteristic plays a significant rôle in the pathology of the disease, finding its most striking manifestation in the rat, where trypanosome development progresses almost unhindered during the later stages of the infection.

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*In a paper which reached us after this paper was prepared for publication, Yorke, Adams and Murgatroyd (1929) also report the 'relatively enormous quantity of glucose consumed by these parasites.' Their figures on glucose consumption are lower than ours, but the general results are the same.

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EFFECT OF THE NATURE AND COMPOSITION OF THE SUBTRATE ON THE DEVELOPMENT AND VIABILITY OF TRYPANOSOMES

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The cultivation of pathogenic trypanosomes has baffled many investigators. Despite periodic reports by various investigators concerning the cultivation of one or another species of pathogenic trypanosome, no suitable method exists which will enable one to cultivate the pathogenic members of this group of organisms with any degree of constancy and certainty. After various unsuccessful attempts to grow these organisms *in vitro*, we have decided to ascertain first the influence of various conditions of the subtrate, such as pH, osmotic pressure, oxygen tension, etc., on their viability and multiplication. It was hoped that basic data of this character would facilitate the ultimate development of a method of cultivation. The object of this paper is to report the results of these studies.

I. EFFECT OF THE pH OF THE MEDIUM ON MULTIPLICATION AND VIABILITY

EXPERIMENT 1. A mixture of 2 c.c. infected rat blood, 6 c.c. normal guinea-pig blood and 6 c.c. Locke solution was prepared. The mixture was divided into two containers, one of which was kept in an atmosphere containing 20 mm., the other 80 mm. CO_2. The respective pH's were 7·16 and 6·64. The temperature was

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24·5° C. Trypanosome counts were made in the blood counting chamber at the beginning of the experiment and at stated intervals thereafter. The results are tabulated below.

Time	Number of tryps. per c.mm. $CO_2 = 20$ mm. Hg. pH $= 7·16$	Number of tryps. per c.mm. $CO_2 = 80$ mm. Hg. pH $= 6·64$
Start	126,000	126,000
After 20 minutes	110,000	90,000
After 60 minutes	69,000	58,000
After 105 minutes	15,000	15,000

EXPERIMENT 2. This experiment was similar to the previous one except that the mixture consisted of 5 c.c. infected rat blood, 5 c.c. guinea-pig blood and 5 c.c. Locke solution, and that the initial number of trypanosomes was smaller. The results are tabulated below.

Time	Number of tryps. per c.mm. $CO_2 = 20$ mm. Hg. pH $= 7·22$	Number of tryps. per c.mm. $CO_2 = 80$ mm. Hg. pH $= 6·66$
Start	35,000	35,000
After 15 minutes	32,000	30,000
After 45 minutes	30,000	29,000
After 75 minutes	29,000	27,000
After 105 minutes	25,000	20,000
After 155 minutes	26,000	17,000

EXPERIMENT 3. In this experiment 2 c.c. infected rat blood, 5 c.c. normal guinea-pig blood and 5 c.c. Locke solution were used ; pH measurements were made at the beginning and end of the experiment. The temperature was 23° C. The results are shown below. (See also Chart I.)

Time	Number of tryps. per c.mm. $CO_2 = 20$ mm. Hg. pH		Number of tryps. per c.mm. $CO_2 = 100$ mm. Hg. pH	
	Initial, 7·52	Final, 6·43	Initial, 6·81	Final, 6·32
Start	88,000		88,000	
After 1 hour	114,000		32,000	
After 2 hours	41,000		8,000	
After 3 hours	10,000		8,000	
After 4 hours	1,500		1,000	
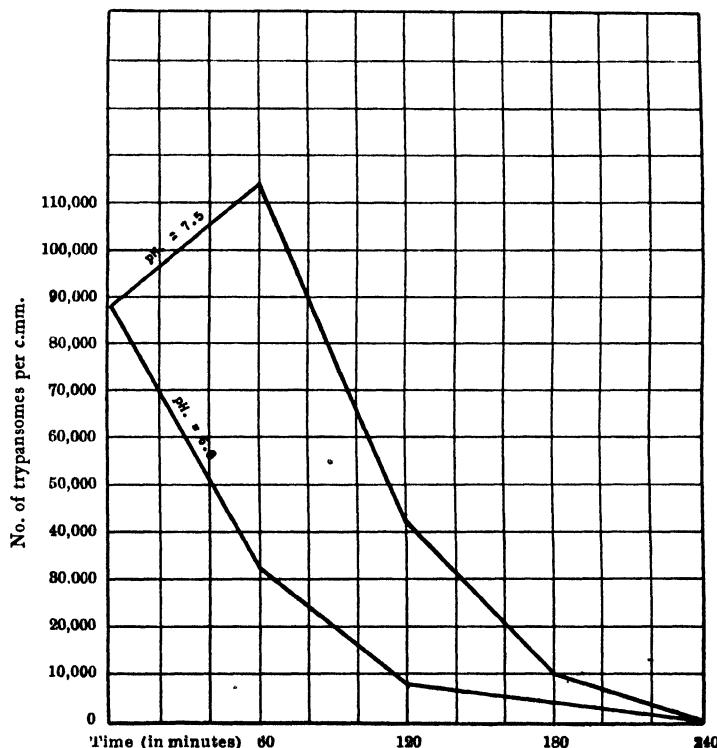
CHART 1. Effect of initial pH on viability and development of trypanosomes. (Experiment 3.)
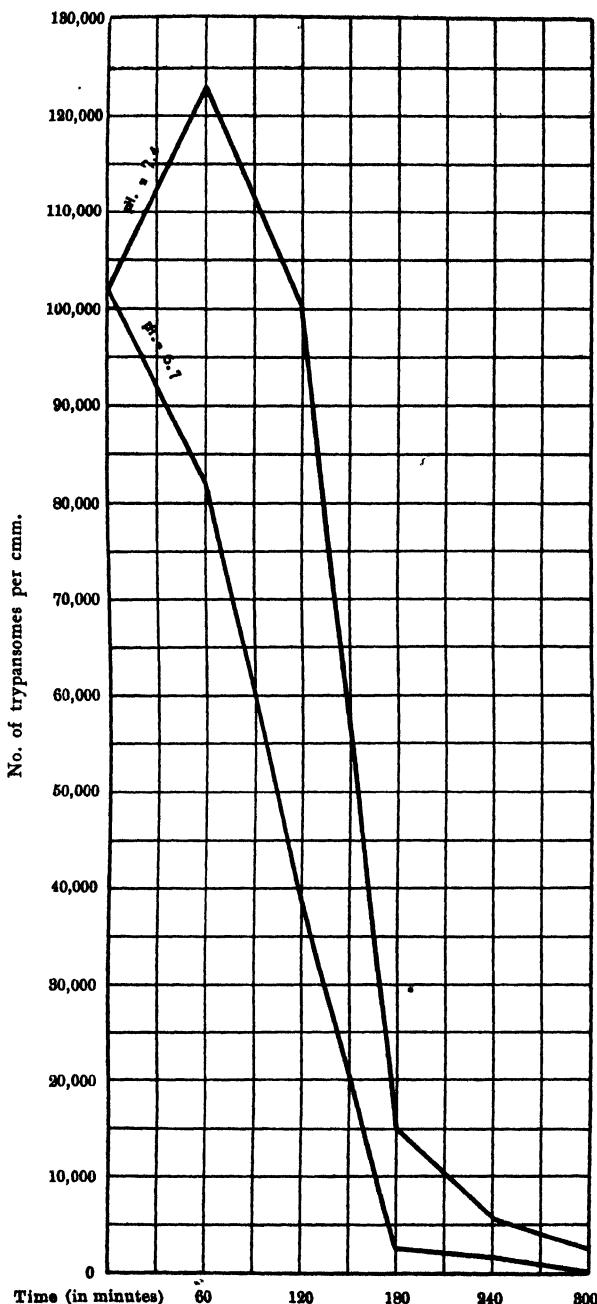
CHART 2. Effect of pH on development and viability of trypanosomes.
(Experiment 4.)

EXPERIMENT 4. In this experiment the mixture consisted of 3 c.c. rat blood, 4 c.c. guinea-pig blood and 5 c.c. Locke solution. Temperature, 24° C. (See also Chart 2.)

Time	Number of tryps. per c.mm. $CO_2 = 20$ mm.	Number of tryps. per c.mm. $CO_2 = 100$ mm.
Start	102,000	102,000
After 1 hour	123,000	82,000
After 2 hours	100,000	39,000
After 3 hours	15,000	2,500
After 4 hours	5,500	1,500
After 5 hours	2,500	0
	Final pH = 6·36	Final pH = 6·24

EXPERIMENT 5. This experiment differed from the previous ones in that four containers, instead of two, were used. The mixture consisted of 5 c.c. rat blood, 5 c.c. guinea-pig blood and 5 c.c. Locke solution. The partial tensions of CO_2 were 10, 40, 80 and 160 respectively. The bicarbonate concentration at the start of the experiment was 10·510 millimol in all containers. Temperature, 24° C. The results are shown below. (See also Chart 2.)

Time	Number of tryps. per c.mm. $CO_2 = 10$ mm. pH = 7·74	Number of tryps. per c.mm. $CO_2 = 40$ mm. pH = 7·13	Number of tryps. per c.mm. $CO_2 = 80$ m.m pH = 6·83	Number of tryps. per c.mm. $CO_2 = 160$ mm. pH = 6·53
Start	22,000	22,000	22,000	22,000
After 1 hour	29,000	25,000	16,000	9,000
After 2 hours	17,000	15,000	11,000	8,000
After 3 hours	20,000	14,500	13,000	3,000
After 4 hours	15,000	13,500	10,500	3,000
After 5 hours	16,000	17,000	3,000	0
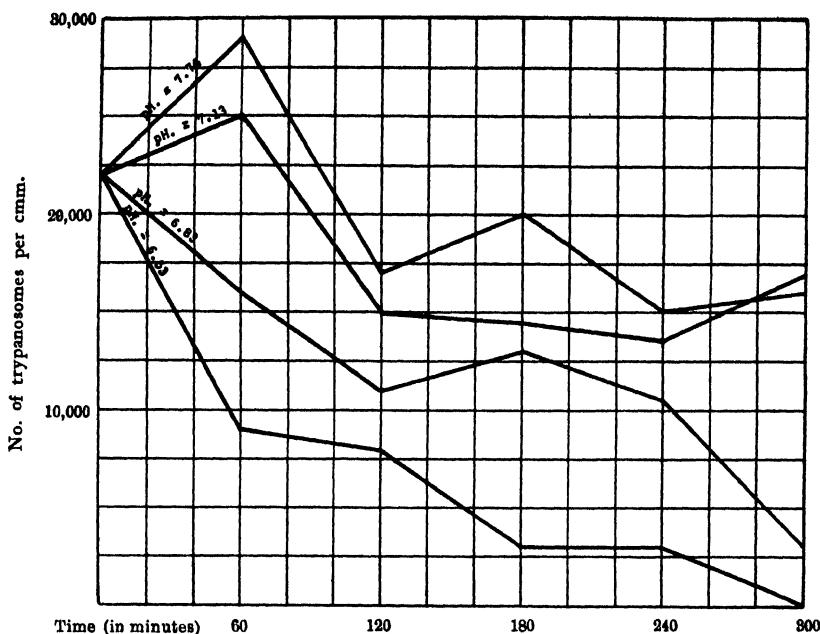
CHART 3. Viability and development of trypanosomes in media of different reactions.

(Experiment 5.)

In the following series the bicarbonate buffer was replaced with phosphate buffers of different pH.

EXPERIMENT 6. The mixture consisted of 1·8 c.c. phosphate buffer m/17, 0·2 c.c. infected rat blood, and glucose solution to give a concentration of 0·4 per cent. Temperature, 24·5 °C.

Time	Number of tryps. per c.mm.	pH	Number of tryps per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH
Start	41,000	6·81	57,000	6·98	30,000	7·17	46,000	7·38
After 1 hour... ...	34,000	...	44,000	...	23,000	...	21,000	...
After 2 hours ...	19,000	...	35,000	...	19,000	...	35,000	...
After 3 hours ...	8,000 .	...	23,000	...	24,000	...	26,000	...
After 4 hours ...	7,000	6·39	10,000	6·44	14,000	6·49	19,000	6·56

EXPERIMENT 7. Mixture of phosphate buffer m/17, containing 0·4 per cent. glucose. To each 3 c.c. of the solution, 0·1 c.c. of infected rat blood was added.

Time	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH
Start	6,000	6·5	7,000	6·8	6,000	7·2	5,000	7·7	3,000	8·0
After 2 hours ...	8,000	...	6,000	...	6,000	...	7,000	...	2,000	...
After 3 hours ...	7,000	...	3,000	...	2,000	...	7,000	...	3,000	...
After 4 hours ...	5,000	...	4,000	...	3,000	...	3,000	...	5,000	...
After 5 hours ...	4,000	...	3,000	...	3,000	...	4,000	...	5,000	...
After 24 hours ...	0	...	0	...	200	...	200	...	3,000	...
After 48 hours ...	0	6·3	0	6·3	0	6·3	cont.	...	cont.	...

EXPERIMENT 8. The mixture consisted of 2 c.c. phosphate buffer m/17, containing 0·4 per cent. glucose and 0·1 c.c. of rat blood, containing 216,000 tryps. per c.mm.

Time	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH
Start	8,000	6·5	8,000	7·0	7,000	7·4	9,000	8·0
After 1 hour ...	11,000	...	6,000	...	6,000	...	11,700	...
After 2 hours ...	8,500	...	4,400	...	8,100	...	11,600	...
After 3 hours ...	8,200	...	6,400	...	5,900	...	12,100	...
After 4 hours ...	8,100	...	6,700	...	7,000	...	7,000	...
After 24 hours ...	0	...	1,300	...	2,400	...	4,000	...
After 30 hours ...	0	6·34	200	6·34	700	6·42	2,200	6·59

EXPERIMENT 9. The mixture consisted of phosphate buffer m/17, containing 0·4 per cent. glucose. To each 3 c.c. of the mixture, 0·3 c.c. of rat blood was added. The results are tabulated below.

Time	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH	Number of tryps. per c.mm.	pH
Start	87,000	6·5	80,000	6·8	61,000	7·0	76,000	7·2
After 1 hour ...	103,000	...	88,000	...	70,000	...	60,000	...
After 2 hours ...	41,000	...	37,000	...	41,000	...	35,000	...
After 3 hours ...	1,000	...	10,000	...	12,000	...	16,000	...

The protocols presented above demonstrate two important points. It seems quite clear that the limiting pH for this species of trypanosome (*T. evansi*) lies around 6·3. As a consequence of this, the initial pH is of profound importance in the development of trypanosomes. Apparently a pH on the acid side is unfavourable to trypanosomes, the buffering effect against acids is less than in alkaline media, and consequently the destruction is continuous and progressive. When the reaction of the medium is alkaline there is first a multiplication followed by death; this is probably due to the rapid elaboration of lactic acid by the fermentation of glucose (Geiger, Kligler and Comaroff, 1930). In all cases the final limiting pH is 6·3 to 6·4, but inhibition and destruction begins at less acid reactions. The optimum evidently lies on the alkali side, but the rapid acidification renders it difficult to maintain an alkali or neutral reaction.

II. EFFECT OF THE OSMOTIC PRESSURE OF THE MEDIUM ON VIABILITY

The effect of salt concentration or osmotic pressure is shown in the following series of experiments. In all of these experiments we have kept the initial reaction and the amount of buffer constant, varying only the salt concentration. The results show that the trypanosome is sensitive to the tonicity of the medium and that the optimum concentration is a 0·07 to 0·1 molar solution, or rather less than the physiological concentration. A reduction or increase in the salt concentration is accompanied by a more rapid diminution in the number of trypanosomes.

EXPERIMENT 1. Phosphate buffer m/15 was diluted with different concentrations of NaCl. in proportion 1 : 1. The phosphate buffer as well as the NaCl. solutions contained 0·4 per cent. glucose. The concentration of NaCl. in the various solution is expressed in fractions of a mol. Initial reaction = pH 8·00. Temperature = 22° C.

Time	Concentration of NaCl.					
	0·035 m.	0·068 m.	0·10 m.	0·167 m.	0·234 m.	0·299 m.
		Number of trypanosomes per c.mm.				
Start	16,000	23,000	24,000	15,000	14,000	19,000
After 1 hour	8,000	8,000	19,000	10,000	13,000	10,000
After 1½ hours	3,000	10,000	22,000	9,000	8,000	10,000
After 2½ hours	500	14,000	7,000	5,000	10,000	6,000
After 3½ hours	...	11,000	7,000	5,000	1,000	700
After 4 hours	...	8,000	9,000	6,000	1,100	500
After 5 hours	...	17,000	10,000	4,000	0	0
After 5½ hours	...	10,000	7,000	0	0	0

EXPERIMENT 2. In this experiment instead of NaCl., Locke solutions in various concentrations were used. The Locke solutions were diluted with phosphate buffer m/15 in the ratio of 3 : 1. The initial pH = 7·4. Temperature = 22° C.

Time	Concentration of solution					
	0·5 Locke	0·75 Locke	1·0 Locke	1·2 Locke	1·4 Locke	1·6 Locke
		Number of trypanosomes per c.mm.				
Start	29,000	16,000	25,000	17,500	16,000	24,000
After 2 hours	15,000	16,000	16,000	14,000	12,000	20,600
After 4 hours	20,800	17,000	18,100	13,500	13,200	19,600
After 24 hours	300	700	100	0	0	0

EXPERIMENT 2a. Same as above, except that Locke solution without buffer was used.

	Concentration of solution					
Time	0·25 Locke	0·5 Locke	1·0 Locke	1·2 Locke	1·4 Locke	1·6 Locke
			Number of trypanosomes per c.mm.			
Start	19,000	12,000	19,000	14,000	11,000	14,000
After 2 hours	12,000	17,000	17,000	6,000	3,000	3,000
After 4 hours	0	19,000	0	0	0	0

III. EFFECT OF LACTATE ION ON VIABILITY

In view of the fact that trypanosomes produce a large amount of lactic acid, it seemed desirable to ascertain whether the lactate ion as such, apart from the increase in $H^{\cdot}$, had an unfavourable effect on the trypanosomes. The following protocols present a number of experiments bearing on this point.

EXPERIMENT 1. The medium consisted of 3·0 c.c. infected rat blood, 6 c.c. normal guinea-pig blood and 6 c.c. of Locke solution. To 5 c.c. of this mixture 0·5 c.c. of n/5 NaCl. solution was added, and to another 5 c.c., 0·25 c.c. of n/5 natrium-lactate and 0·25 c.c. n/5 NaCl. were added. pH = 7·4; CO_2 = 40 m.m.; Temperature = 24° C.

Time	Na-Lactate	Na-Chloride
	Number of trypanosomes	Number of trypanosomes
Start	47,000	47,000
After 1 hour	50,000	27,000
After 2 hours	4,500	9,500

EXPERIMENT 2. The basic mixture was the same as above ; to one portion of 5 c.c. of this mixture, 0·5 c.c. n/5 lactate, to the other 0·2 c.c. n/5 lactate, and to the third 0·5 c.c. n/5 NaCl. were added. Reaction pH = 7·4 ; Temperature = 24·5 °C.

Time	Na-Lactate 0·5 c.c.	Na-Lactate 0·2 c.c.	NaCl. 0·5 c.c.
	Number of trypanosomes per c.mm.	Number of trypanosomes per c.mm.	Number of trypanosomes per c.mm.
Start	44,500	69,000	28,000
After 1 hour ...	18,500	24,500	7,000
After 2 hours ...	18,000	22,500	5,000
After 3 hours ...	12,500	cont.	2,000

EXPERIMENT 3. The subtrate consisted of 2 c.c. rat blood, 5 c.c. normal guinea-pig blood and 5 c.c. Locke solution, to which was added Na-Lactate solution in desired proportion. The results are tabulated below.

Time	N/50 Lactate	N/10 Lactate	N/10 NaCl.
	Number of trypanosomes	Number of trypanosomes	Number of trypanosomes
Start	140,000	140,000	140,000
After 1½ hours ...	101,000	96,500	70,000
After 2½ hours ...	26,000	15,000	4,500
After 3½ hours ...	12,500	9,000	1,000
After 4½ hours ...	5,500	9,000	1,000
After 5½ hours ...	2,500	5,500	0

EXPERIMENT 4. Same as above. To one portion of 5 c.c., 0·5 c.c. n/5 NaCl. solution, to the other portion of 5 c.c., 0·5 c.c. of serum of a highly infected rat (670,000 tryps. per c.mm.), and to a third portion of 5 c.c., 0·25 c.c. n/5 Na-Lactate were added.

Time	NaCl. Number of trypanosomes per c.mm.	Lactate Number of trypanosomes per c.mm.	Serum Number of trypanosomes per c.mm.
Start	47,000	47,000	47,000
After 1 hour ...	27,000	50,000	76,000
After 3 hours ...	4,500	20,000	9,500

These experiments indicate that the lactate ion as such has no unfavourable effect. On the contrary, it is apparent that the chlorine ion is more injurious than is the lactate. It would seem that the most important inhibiting factor is the hydrogen ion concentration.

IV. EFFECT OF GLUCOSE CONCENTRATION ON VIABILITY

Schern (1925) has shown that the viability of trypanosomes is influenced by the glucose concentration. He noted the rapid death of trypanosomes in the blood of heavily infected rats and attributed it to the exhaustion of sugar. He reported that adding sugar to the blood leads to a revival of trypanosomes and an increased mobility. Although the experiments reported above suggest that the deleterious effect was due to the acid, it was nevertheless important to ascertain the effect of different sugar concentrations on the viability of trypanosomes.

A few typical experiments are presented below. It appears that although concentrations of glucose below 0·1 per cent. are unfavourable, higher concentrations are not more favourable.

EXPERIMENT 1. Phosphate buffer m/17, pH = 7·4 plus various concentrations of glucose. To each 3 c.c. of the mixture 0·25 c.c. infected rat blood were added.

Time	0·01% glucose Number of tryps. per c.mm.	0·03% glucose Number of tryps. per c.mm.	0·06% glucose Number of tryps. per c.mm.	0·20% glucose Number of tryps. per c.mm.
Start	135,000	155,000	138,000	69,000
After 1 hour	19,000	12,000	113,000	48,000
After 2½ hours ...	...	4,000	2,000	30,000
After 3½ hours ...	400	1,000	800	39,000
After 4 hours ...	0	0	0	24,000

EXPERIMENT 2. Phosphate buffer m/17, pH = 7·38. To each 3 c.c. of the mixture, 0·15 c.c. rat blood was added.

Time	0·02% glucose Number of tryps. per c.mm.	0·1% glucose Number of tryps. per c.mm.	0·2% glucose Number of tryps. per c.mm.	0·4% glucose Number of tryps. per c.mm.
Start	35,000	38,000	35,000	31,000
After 1 hour	21,000	16,000	23,000	27,000
After 2 hours	3,500	20,000	14,000	14,500
After 3 hours	3,500	20,000	14,000	14,500
After 4 hours	0	13,000	8,000	17,000

V. EFFECT OF PARTIAL PRESSURE OF O_2 ON VIABILITY

The final point to determine was the effect of the O_2 tension. It is evident from a large series of experiments that the development of trypanosomes is dependent on the oxygen tension. In the absence of oxygen the development is retarded. In the presence of atmospheric O_2 tension, the development is rapid and the limiting pH is soon reached. The result is, therefore, a paradoxical one, a longer survival in the absence of O_2.

Some of the experimental data are presented in the following protocols.

EXPERIMENT 1. To 2 c.c. of a mixture of equal portions of phosphate m/17 and Locke solution was added 0·05 c.c. of infected rat blood. The solution contained 0·4 per cent. glucose. One set was kept in the air, the other under nearly complete anaerobic conditions. pH = 7·6. Temperature = 23·5° C.

Time	O_2 = atmospheric pressure Number of trypanosomes per c.mm.	Oxygen replaced by Nitrogen Number of trypanosomes per c.mm.
Start	21,800	32,200
After 1 hour	34,900	28,200
After 2 hours	36,400	32,500
After 4 hours	18,000	48,000

EXPERIMENT 2.   The same as above.   pH = 7·5.

Time	O₂ = atmospheric pressure	Oxygen replaced by Nitrogen
	Number of trypanosomes per c.mm.	Number of trypanosomes per c.mm.
Start	29,700	23,500
After 1 hour	31,500	35,500
After 3 hours	31,500	41,000
After 4 hours	32,100	40,200

EXPERIMENT 3.   The same as above.   Phosphate buffer m/15. pH = 7·4.

Time	O₂ = atmospheric pressure	Oxygen replaced by Nitrogen
	Number of trypanosomes per c.mm.	Number of trypanosomes per c.mm.
Start	36,000	38,000
After 24 hours	700	13,000
After 48 hours	0	12,000
After 56 hours	0	6,000

EXPERIMENT 4.   The same as above, except that the initial pH varied in each case.

Time	O₂ atmospheric pressure pH = 6·76	Oxygen replaced by Nitrogen initial pH = 6·76	O₂ atmospheric pressure pH = 7·64	Oxygen replaced by Nitrogen initial pH = 7·64
	Number of tryps. per c.mm.	Number of tryps. per c.mm.	Number of tryps. per c.mm.	Number of tryps. per c.mm.
Start	8,500	7,500	7,000	9,000
After 1 hour ...	10,500	6,000	5,900	11,700
After 2 hours ...	8,500	4,400	8,100	11,600
After 3 hours ...	8,200	6,400	5,900	12,100
After 4 hours ...	8,100	6,700	7,000	.7,000
After 24 hours ...	0	1,300	2,400	4,000
After 30 hours ...	0	200	700	2,200

Further evidence of the aerobic character of the trypanosomes is furnished by the data in the following table. In these experiments we determined the lactic acid production in identical media under aerobic and anaerobic conditions respectively. It will be noted that the lactic acid production under aerobic conditions is three to four times as high as in the absence of oxygen.

TABLE I

Lactic acid production by *Trypanosome evansi* in the presence and absence of air.

Date	Temp., ° C.	CO_2 tension, mm. Hg.	Atmospheric O_2 pressure							
			Average number of tryps. per c.mm.	Duration of experiment (in mins.)	Millimol CO_2 per litre at start	Millimol CO_2 per litre at end	Millimol CO_2 per litre evolved	Lactic acid produced mgm. %	Mgm. lactic acid produced by 1 billion tryps. per hour	Initial pH
15.ix	24	20	15,000	120	13·96	11·96	2·00	18·00	6·0	7·50
	24	20	31,000	120	14·37	10·68	3·96	33·20	5·3	7·48
	24	20	24,000	120	14·01	11·34	2·67	24·03	5·0	7·50
18.ix	24	80	8,500	180	14·465	13·711	0·74	6·666	2·61	6·84
19.ix	24	80	30,500	120	12·966	11·031	1·935	17·43	2·49	6·74

Date	Temp., ° C.	CO_2 tension, mm. Hg.	Without Oxygen							
			Average number of tryps. per c.mm.	Duration of experiment (in mins.)	Millimol CO_2 per litre at start	Millimol CO_2 per litre at end	Millimol CO_2 per litre evolved	Lactic acid produced mgm. %	Mgm. lactic acid produced by 1 billion tryps. per hour	Initial pH
15.ix	24	20	19,000	150	14·30	13·98	0·32	2·88	1·51	7·54
	24	20	41,000	120	13·64	12·57	1·07	9·63	1·16	7·50
	24	20	29,000	180	14·02	12·27	1·75	15·75	1·80	7·50
18.ix	24	80	16,000	180	14·462	14·08	0·379	3·41	0·71	6·85
19.ix	24	80	29,500	120	12·881	12·390	0·491	4·42	0·75	6·77

NOTE.—The experiments were carried out in duplicate, one with the other without oxygen.

SUMMARY

Summarizing the results of our experiments it appears that the most important single factor responsible for the difficulty experienced in cultivating these organisms is their active production of lactic acid. Since these organisms are aerobic and require a certain glucose concentration for survival, the fermentation of the glucose leads to the production of lactic acid, which in the ordinary buffered solutions rapidly reaches a concentration inhibitive and apparently even toxic to the cell. This accounts for the frequent observation that in blood drawn from a heavily infected animal, the trypanosomes quickly become clumped and die, giving the impression of agglutination. What actually happens is that the large number of trypanosomes requires very little time to produce a limiting acidity in blood already having a higher lactic acid content (Kligler, Geiger and Comaroff, 1929) and lower alkali reserve than normal.

There is reason to believe that the data presented above will add to our understanding of the physiology of this group of parasites and help towards the elaboration of suitable methods for cultivating them. Any method must take into account the fact that the zone of tolerance of H· is narrow, and that the trypanosomes actively ferment glucose with the production of lactic acid. The other factors, glucose concentration, osmotic pressure, toxicity of NaCl, are naturally important, but nowhere near as significant. It is doubtful whether prolonged cultivation without frequent, if not daily, passage will be possible.

CONCLUSIONS

The results of our experiments suggest the following conclusions :—

1. *T. evansi* is sensitive to hydrogen ion concentration. The optimum pH is on the alkali side (probably 7·4) ; the limiting pH is between 6·3 to 6·4 ; while the inhibiting pH is probably near 6·8.

2. *T. evansi* requires a minimum concentration of glucose, about 0·1 per cent. In the presence of oxygen the glucose is rapidly fermented with the production of lactic acid.

3. *T. evansi* is aerobic ; it survives longer under anaerobic than under aerobic conditions, but activity is greatly inhibited as evidenced by the acid production.

4. The optimal osmotic pressure is that of the tissues rather than that of the blood ; 0·08/m to 0·1/m or $\frac{3}{4}$ physiologic solution being more favourable than 0·12/m.

5. NaCl. is more toxic than Na-Lactate ; the inhibitive effect of lactic acid is due, therefore, to the hydrogen ion concentration rather than the lactate.

Since the completion of these experiments, Yorke, Adams and Murgatroyd (1929) published data which emphasise the significance of these results. These authors found that the survival of trypanosomes for any length of time is conditioned by their number. If the initial number of trypanosomes is large they die more rapidly than when the initial number is small. This difference is undoubtedly due to the more rapid acidification of the medium by the large number of organisms.

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EXPERIMENTS WITH *DIROFILARIA IMMITIS* AND LOCAL SPECIES OF MOSQUITOS IN PEIPING, NORTH CHINA

WITH A NOTE ON *LANKESTERIA CULICIS* FOUND IN *AEDES KOREICUS**

BY

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PLATES XI AND XII

It is well known that dogs in North China are very frequently infected with *Dirofilaria immitis*, but, up to the present, no observations seem to have been made as to what species of mosquitos transmit this parasite. In the autumn of 1929, I had an opportunity to carry out a series of experiments in Peiping, North China, from the results of which it appears that at least two species of mosquitos, namely, *Anopheles hyrcanus* var. *sinensis* and *Aedes koreicus*, are responsible for the transmission of this parasite in this area.

METHODS AND TECHNIQUE

Seventeen species of mosquitos were listed by Meleney, Lee and Yang (1928) in the vicinity of Peiping, but the most common ones are *Aedes koreicus*, *Culex pipiens*, *Anopheles hyrcanus* var. *sinensis* and *Anopheles* (*Myzomyia*) *pattoni*. These experiments, therefore, were made mainly with these species but occasionally some other species were obtained.

Different species of mosquito larvae were collected in and around Peiping and bred out in the laboratory. The mosquitos when

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hatched out were left in the cage for a day or so before feeding to starve them so that they might feed more readily. They were then placed in feeding-boxes similar to those used in the malaria experiments by Hindle and Feng (1929). The dog used was a tame one which was heavily infected with *Dirofilaria immitis*. Frequent examinations showed that in a very small drop of blood under an ordinary-sized cover slip the Microfilariae averaged 45 in number. The dog's hair was removed by barium sulphide paste, and the skin carefully washed and dried before feeding the mosquitos. The feeding boxes containing the mosquitos were then put on the skin of the dog and the mosquitos were allowed to feed for half an hour, after which they were freed in a cage. Those which had fed were put in earthen pots which were standing in a basin of water to keep them moist and the temperature was kept between 25° and 28° C. These mosquitos were then fed on a rabbit daily to keep them alive. Since the purpose of these experiments was to see whether or not development of *Microfilaria immitis* could take place and be completed in the local species of mosquitos, none of them was killed until the larvae of *D. immitis* were suspected to be present in the labium. When any of the mosquitos died, however, in the course of the experiment, they were dissected and the malpighian tubes and stomach carefully searched under the microscope for Microfilariae. When they died eight or more days after feeding, the thorax, head and proboscis were also carefully examined for Microfilariae in addition to the usual examination of malpighian tubes and stomach. In some cases, selected specimens were preserved in Bless' fluid and sections made.

EXPERIMENTS WITH *AEDES KOREICUS*

The mosquitos used in this experiment were all bred out from larvae and pupae of *Aedes koreicus* collected from large water kongs in the Central and Pei Hai Parks of Peiping during the late summer. The results are listed in the following table (Table I) :—

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
K-1 Sept. 9.29 ...	10	1	1	Negative.
	...	3	1	Negative.
	...	4	2	Both negative.
	...	5	1	Positive in malpighian tubes.
	...	8	2	Both positive in malpighian tubes.
	...	9	1	Mature larvae in malpighian tubes.
	...	11	1	Mature larvae in malpighian tubes.
	...	14	1	5 mature larvae in head, 2 in labium.
K-2 Sept. 11.29 ...	4	3	1	Negative.
	...	4	1	Negative.
	.?.	6	1	Positive in malpighian tubes.
	...	7	1	Negative.
K-3 Sept. 12.29 ...	13	1	2	Both negative.
	...	2	2	One negative and one positive in malpighian tubes.
	...	3	3	One positive in malpighian tubes and two negatives.
	...	5	1	Positive in malpighian tubes.
	...	6	1	Positive in malpighian tubes.
	...	11	1	Two mature larvae in malpighian tubes and one in labium.
	...	14	1	Mature larvae in malpighian tubes and labium.
	...	...	*2	One mature larva in labium and one negative.
K-4 Sept. 14.29 ...	8	1	4	All negative.
	...	4	1	Negative.
	...	5	2	Both positive in malpighian tubes.
	...	10	1	Mature larvae in malpighian tubes.
K-5 Sept. 16.29 ...	3	3	1	Positive in malpighian tubes.
	...	12	2	One positive in labium ; one negative.

TABLE I—*continued*.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
K-6 Sept. 17.29 ...	9	3	4	One positive in malpighian tubes; three negatives, degenerated.
	...	4	1	Negative, degenerated.
	...	6	1	Positive in malpighian tubes.
	...	9	1	Six premature larvae in malpighian tubes.
	...	11	*1	Positive in labium, thorax, abdominal cavity and malpighian tubes.
	...	13	*1	Positive in thorax.
K-7 Sept. 18.29 ...	12	2	4	Three negatives; one positive in malpighian tubes.
	...	3	1	Negative, degenerated.
	...	4	1	Positive in malpighian tubes.
	...	6	1	Negative.
	...	8	1	Negative, degenerated.
	...	9	1	Positive in malpighian tubes.
	...	10	1	Mature larvae in malpighian tubes only.
	...	...	2	One positive in malpighian tubes; one negative.
	...	12	1	Four mature in malpighian tubes.
K-8 Sept. 20.29 ...	3	8	*1	Positive in malpighian tubes.
	...	10	*2	(a) Mature larvae in labium, head, thorax and abdominal cavity. (b) Positive in abdominal cavity and thorax.
K-9 Sept. 21.29 ...	37	1 hour	*2	Both positive in stomach and cells of malpighian tubes; one showed microfilaria at junction of mid and hind gut.
	...	6 hours	*2	Both positive in stomach and malpighian tubes.
	...	10 hours	*2	(a) Positive in malpighian tubes and stomach. (b) Positive in malpighian tubes only.
	...	15 hours	*2	Both positive in malpighian tubes.
	...	20 hours	*2	Positive in malpighian tubes.
	...	1	*2	Positive in malpighian tubes.
	...	1	1	Negative.
	...	3	5	Three positives in malpighian tubes; two negatives (degenerated).
	...	4	2	Both positive in malpighian tubes.
	...	5	4	Two positive in malpighian tubes; two negatives.
	...	6	10	Five negatives (degenerated). Five positives in malpighian tubes; one fly contained fourteen microfilariae in malpighian tubes.
	...	9	3	All positive in malpighian tubes (5-9 premature larvae in each fly).

TABLE I—*continued*.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
K–10A Sept. 23.29 ...	30	2	2	Positive in malpighian tubes.
	...	3	5	Three negatives (degenerated); two positives in malpighian tubes.
	...	6	7	Three positives in malpighian tubes (calcified larvae in one fly); four negatives (two degenerated).
	...	7	8	Five positives in malpighian tubes; three negatives.
	...	8	2	Both negatives (one degenerated).
	...	9	3	Two positives in malpighian tubes; one positive (mature larvae) in thorax and abdominal cavity.
	...	15	*3	(a) Three mature larvae in labium, and one in head. (b) Mature larvae in labium, head, thorax, abdominal cavity and malpighian tubes. (c) Negative.
K–10B Sept. 23.29 ...	29	4	3	All positive in malpighian tubes.
	...	5	6	Two negatives; four positives in malpighian tubes (all showed both alive and calcified larvae).
	...	6	3	One positive; two negatives (degenerated).
	...	7	11	Six positives in malpighian tubes; five negatives (four degenerated).
	...	8	1	Six mature larvae in malpighian tubes.
	...	11	1	Eight mature larvae in malpighian tubes and abdominal cavity.
	...	13	*1	Mature larvae in labium, head, thorax, abdominal cavity and malpighian tubes.
	...	15	1	Fly found dead, negative in all parts.
	...	...	*2	(a) Five mature larvae in labium, one in head and one calcified in malpighian tubes. (b) Two mature larvae in labium, one in head and two in malpighian tubes.
K–11 Sept. 24.29 ...	11	4	1	Positive in malpighian tubes.
	...	6	5	Two negatives; three positives (in one of these flies ten calcified and three living larvae were found).
	...	7	2	One negative; one positive.
	...	13	1	Mature larvae in labium, head and abdominal cavity.
	...	14	*2	(a) Two mature larvae in labium and labella; two in head and one in abdominal cavity. (b)*Two mature larvae in labium.

TABLE I—*continued*.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
K–12 Sept. 25.29 ...	16	4	3	Two negatives; one positive, alive and calcified dead larvae in malpighian tubes.
	...	5	3	One negative; two positives in malpighian tubes.
	...	6	6	Two negatives; four positives (in one of these flies seven living and fifteen calcified larvae found in malpighian tubes).
	...	7	2	(*a*) Calcified larvae in malpighian tubes. (*b*) One negative.
	...	8	1	Both living and calcified dead larvae in malpighian tubes.
	...	9	1	Negative.
K–13 Oct. 2.29 ...	1	7	1	Positive in malpighian tubes.

A total of 187 mosquitos of this species (*Aedes koreicus*) were fed on the dog. Of this number 100 were positive (55 per cent.). Most of the negative ones were dissected in the early days and many of the mosquitos were so disorganised that even if the small Microfilariae were present they were too degenerated to be recognized. From the tenth to the fifteenth day after feeding twenty-seven mosquitos were dissected, of which all but four were positive (85 per cent.). Of the 23 that were positive, larvae were found in the labium in 15, and in the remaining 8 they were seen in the malpighian tubes.

From this it may be concluded that if the mosquitos had lived as long as they probably would have under natural conditions, the majority would have been positive for *Microfilaria immitis* and capable of transmitting this parasite.

The Microfilariae begin to leave the stomach and creep into the malpighian tubes about one hour after feeding, and by the end of the fifteenth hour the process is completed. As soon as they reach the malpighian tubes, they penetrate the cells of these tubes and live in them, but not in the lumen (see Pl. XI, fig. 2).

The development of Microfilariae in the mosquitos depends on the temperature (see also later in experiments with *Anopheles*

hyrcanus, var. *sinensis*). At a temperature of 25°-28° C. it takes about two days for the larvae to change into short-tailed, sausage-shaped forms. Later when the worms look very thick and stumpy the internal organs gradually become visible. As the Microfilariae develop, they become thinner and longer again and from the tenth to fifteenth day (usually at the twelfth day) they become long, filiform, actively moving, mature larvae, measuring on an average 33·2μ × 99·8 μ. They then rupture the cells of the malpighian tubes, become free, and wander about in the abdominal cavity of the mosquito. They migrate in different directions, but the majority finally reach the labium and come out from the labella (Pl. XI, figs. 1-A and 1-B). Some may go into the legs, others into the brain and above the pharynx. Some die in the course of development, as is shown by the calcification of Microfilariae in the malpighian tubes which took place at different stages of development (Pl. XI, figs. 3 and 4). In some cases all the larvae in the malpighian tubes become calcified and in others some calcify, while some are alive and continue their development. In fresh dissections, the calcified larvae appear as black rigid dead worms (Pl. XI, fig. 3).

In the course of dissection of this species of mosquito (*Aedes koreicus*), several instances of infection with gregarine *Lankesteria culicis* were encountered, about which a short note is given at the end of this paper.

EXPERIMENTS WITH *ANOPHELES HYRCANUS* var. *SINENSIS*

This species of Anopheles is very common all over China. In Peiping they breed in great numbers in September and October in ponds where vegetation is abundant, but they are seldom found, if at all, in fresh water in brooks among the hills. Mosquitos used in this experiment were bred out in the laboratory from larvae collected from the ponds in Haitien, about four miles north-west of Peiping. The results are listed in Table II.

Altogether 126 *Anopheles hyrcanus* were fed on the dog. Deducting 12 which fed on the puppy for the purpose of transmission, 78 of the remaining 114 were positive for *Microfilaria immitis* (68 per cent.). There were 53 which died after the tenth day, 37 of which were positive (70 per cent.). From the above data it is clear that *Microfilaria immitis* can also complete its

development in this species of Anopheles. The development of *Microfilaria immitis* in this species of mosquito was similar to that in *Aedes koreicus* but slower, taking about fifteen days for the Microfilariae to reach the labium. This was probably due to the fact that the season was rather late and that the temperature in the laboratory, although it was heated, was not very high, especially at night, when the temperature fell to about 20° C. Dead and calcified Microfilariae were not seen in this species of mosquito. The mature Microfilariae were seen proceeding to the various parts of the body, as noted in *Aedes koreicus*. In addition, the mature embryos were in one case seen in the palpi.

TABLE II

Result of Experiments with *Anopheles hyrcanus* var. *sinensis*

(Kept at 25°–28° C.).

*Fixed in Bless' fluid and sectioned.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
H-1 Sept. 26.29 ...	4	4	1	Positive in malpighian tubes.
	...	5	2	Both positive in malpighian tubes.
	...	8	1	Positive in malpighian tubes.
H-2 Sept. 27.29 ...	10	5	1	Positive in malpighian tubes.
	...	6	2	One positive in malpighian tubes; one degenerated negative.
	...	10	2	Both positive; one containing fifteen premature larvae in malpighian tubes and one containing three larvae.
	...	11	3	Two positives and one negative.
	...	12	2	Both positive (mature larvae in malpighian tubes.)
H-3 Sept. 30.29 ...	5	3	1	Positive in malpighian tubes.
	...	4	2	One positive in malpighian tubes and one negative.
	...	8	2	Both positive in malpighian tubes.

TABLE II—*continued*.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
H-4 Oct. 19.29 ...	7	6	1	Positive in malpighian tubes.
	...	8	1	Positive in malpighian tubes (premature forms).
	...	10	1	Positive in malpighian tubes (premature forms).
	...	11	1	Positive in malpighian tubes (mature form).
	...	12	1	Mature larva in abdominal cavity.
	...	16	•1	Negative.
	...	16	•1	Negative.
H-5 Oct. 21.29 ...	21	3	1	Negative.
	...	6	2	One negative and one positive in malpighian tubes.
	...	7	2	One negative and one positive in malpighian tubes.
	...	8	1	Positive in malpighian tubes.
	...	9	2	Both positive in malpighian tubes (premature larvae).
	...	10	3	Two negatives and one positive.
	...	11	3	All positive in malpighian tubes.
	...	12	1	Positive in malpighian tubes.
	...	13	1	Negative.
	...	13	5	Feeding on young puppy (No. 1) and found negative after dissection.
H-6 Oct. 21.29. ...	20	4	1	Positive in malpighian tubes.
	...	6	2	Both positive in malpighian tubes.
	...	8	2	One positive and one negative (premature).
	...	9	1	Negative (degenerated.)
	...	11	3	Two positives (premature); one negative (degenerated).
	...	12	2	Both positive in malpighian tubes (mature).
	...	13	7	Feeding on puppy No. 2 daily for three days, when died all dissected; four positives and three negatives.
H-7 Oct. 21.29 ...	9	8	1	Positive in malpighian tubes.
	...	12	1	Mature larvae in malpighian tubes.
	...	13	7	Feeding on puppy No. 1 daily. 2 days later, three dissected, in one of which mature larvae were found in abdominal cavity only. Two negatives. One died on 3rd, 4th and 7th days respectively, dissection all negative. One escaped.

TABLE II—*continued*.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
H-8 Oct. 21.29 ...	24	3	1	Positive in malpighian tubes.
	...	6	2	One positive and one negative (degenerated).
	...	7	2	Both negative.
	...	9	1	Positive in malpighian tubes.
	...	10	3	One positive and two negatives.
	...	12	1	Positive.
	...	14	1	Negative.
	...	15	8	Four negatives (two degenerated); two with mature larvae in labium; two with mature larvae in malpighian tubes and abdomen.
	...	...	*5	Two positives in head and labium; three negatives.
H-9 Oct. 22.29 ...	29	3	1	Positive in malpighian tubes.
	...	5	6	Two positives; four negatives (degenerated).
	...	6	3	All positive.
	...	7	4	All positive.
	...	8	2	One positive and one negative.
	...	11	1	Positive.
	...	12	4	Two positives (one containing fifteen microfilariae in malpighian tubes); two negatives (degenerated).
	...	14	3	All positive.
	...	15	2	Both positive in head, proboscis and abdomen.
	...	15	*2	Both positive in malpighian tubes, abdomen and proboscis.
	...	16	*1	Positive in malpighian tubes only.
H-10 Oct. 22.29 ...	6	1	1	Positive in stomach.
	...	3	1	Positive in malpighian tubes.
	...	5	1	Positive in malpighian tubes.
	...	8	1	Negative.
	...	10	1	Positive in malpighian tubes.
	...	12	1	Positive in malpighian tubes.

EXPERIMENTS WITH *ANOPHELES MYZOMYIA PATTONI*

At the end of September and in later months, larvae of *A. myzomyia pattoni* were found in great numbers in the small pools and streams in the Western Hills about eight miles west of Peiping. These were collected and bred in the laboratory, the bred mosquitos being fed in the same way as the others. The following is a table of the result.

TABLE III

Result of experiments with *Anopheles myzomyia pattoni*

(Kept at 25°–28° C.)

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
P–1 Sept. 13.29 ...	6	2	1	Negative.
	...	4	1	Negative.
	...	5	3	All negative, one degenerated.
	...	7	1	Negative.
P–2 Sept. 14.29 ...	2	1	1	Negative.
	...	3	1	Negative.
P–3 Sept. 23.29 ...	8	2	1	Negative (degenerated).
	...	8	1	Negative.
		9	1	Negative.
	...	10	5	All negative.
P–4 Sept. 24.29 ...	4	5	2	Negative.
	...	7	1	Negative.
		16	1	Negative.
P–5 Sept. 22.29 ...	1	8	1	Negative.
P–6 Oct. 14.29 ...	1	3	1	Negative.
P–7 Oct. 16.29 ...	1	14	1	Negative.
P–8 Oct. 18.29 ...	17	3	7	All negative.
	...	4	3	All negative.
	...	5	4	All negative.
	...	10	1	Negative.
	...	15	1	Negative.
P–9 Oct. 19.29 ...	22	1	1	Negative. (See section).
	...	2	1	Negative.
	...	3	3	All negative.
	...	4	5	All negative.
	...	5	2	Both negative.
	...	8	4	All negative.
	...	9	1	Negative.
	...	10	2	All negative.
	...	12	2	Both negative.
	...	13	1	Negative.

TABLE III—*continued*.

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
P–10 Oct. 22.29 ...	5	5 12 13	2 1 2	Both negative. Negative. Both negative.
P–11 Oct. 28.29 ...	3 ...	2 3	1 2	Negative. Negative.
P–12	10	at once	10	Three positives, one to three microfilariae. Remainder all negative (some partially fed).

Altogether 80 *A. myzomyia pattoni* were fed on the infected dog. Seventy of these were dissected at intervals ranging from one to sixteen days after feeding, but none was found positive for *Microfilaria immitis*. Ten *A. myzomyia pattoni* were, therefore, fed and killed immediately after feeding (P-12) and the stomach contents carefully examined microscopically in fresh preparations. Three out of the ten were positive (they contained 3, 2, and 1 larvae respectively) It is to be noted that several of these mosquitos took up only a small amount of blood, which might not have contained any larva. Of the 80 fed ones, however, according to this percentage, one should find at least 30 per cent. positive if this species is a suitable intermediate host. In *A. hyrcanus* var. *sinensis*, on the other hand, in some cases after only one feeding on the infected dog, the number of embryos in one mosquito may reach as many as fifteen (Table II, H-9). *A. myzomyia pattoni* is smaller than *A. hyrcanus*, but even if the mosquito should only take half as much blood as *A. hyrcanus*, in some cases it should contain at least five larvae. Since all the *A. pattoni* except for the three killed immediately after feeding, were negative for *Microfilaria immitis*, it seems evident that *A. myzomyia pattoni* is not a suitable intermediate host for this parasite.

EXPERIMENTS WITH *CULEX PIPIENS*

Culex pipiens is the common house mosquito of North China. Larvae were collected from the water-kongs placed at the sides of the streets in Peiping City, and mosquitos were bred out in the laboratory. It is very striking to note that it was difficult to get these mosquitos to feed, less than one per cent. biting the dog. As advocated by MacGregor (1929) a dilute solution of honey was smeared on the skin of the dog, but most of them were found to be only sucking the honey instead of biting the animal. Unfortunately the season was too late to get enough specimens of this species of mosquito to carry on further experiments. Of all the mosquitos fed in one case only several degenerating Microfilariae were found in the stomach one day after feeding. It may be inferred that the Microfilariae probably degenerated in the mosquitos and no development took place.

TABLE IV

Result of experiments with *Culex pipiens*

(Kept at 20°–28° C.)

No. of experiment and date when fed	No. of flies fed	No. of days between feeding and dissection	No. of flies dissected	Result of dissection and remarks
C–1				
Sept. 12.29 ...	2	5	2	Negative.
C–2				
Sept. 13.29 ...	2	5	2	Negative.
Sept. 30.29 ...	1	2	1	Negative.
Oct. 2.29 ...	1	6	1	Negative.
Oct. 14.29 ...	4	2	2	Negative.
...	...	...	1	Negative.
...	...	3	1	Negative (degenerated).
Oct. 15.29 ...	2	1	1	Degenerating microfilariae in stomach.
...	...	2	1	Negative.
Oct. 18.29 ...	2	2	1	Negative.
...	...	4	1	Negative.
Oct. 19.29 ...	10	2	6	All negative.
...	...	4	1	Negative.
...	...	5	1	Negative.
...	...	...	1	Negative.
...	...	8	1	Negative (degenerated).
Oct. 21.29 ...	4	4	1	Negative.
...	...	6	3	All negative.
Oct. 22.29 ...	3	1	1	Negative.
...	...	5	1	Negative.
...	...	9	1	Negative.

EXPERIMENTS WITH OTHER SPECIES OF MOSQUITOS

Two other fairly common species of mosquitos are *Culex tritaeniorrhynchus* and *Luzia vorax*, but few of these mosquitos have been tried. These also did not feed readily on the dog and hence no conclusion can be drawn. Other species of mosquitos were not available for experiments.

SUMMARY AND CONCLUSION

Microfilariae of *Dirofilaria immitis* undergo their development experimentally in *Anopheles hyrcanus* var. *sinensis* and *Aedes koreicus*. The microfilariae pass into the malpighian tubes within one to twenty-four hours after feeding. At a temperature of 25°-28° C. the whole development is completed in ten to fifteen days, and between twelve and fifteen days they proceed forward to the labium. The mature larvae have been seen proceeding to the legs, in the head above the pharynx, and sometimes even in the palpi. At different stages of development, dead and calcified larvae were observed in *A. koreicus*, but not in *A. hyrcanus* var. *sinensis*. Both *A. hyrcanus* and *A. koreicus* are equally good for transmission of the filaria, as dogs in China usually live out of doors and neither of these species often enters a house. From this close association it may be concluded that they play the principal rôle in transmission of this parasite. Of the two species of mosquitos, *A. hyrcanus* var. *sinensis* probably plays a more important part in the transmission, for its distribution is wide throughout China whereas *A. koreicus* is confined to Korea and North China. It is quite possible that other species of Aedes in other parts of China, such as *Aedes albopictus*, the most common Aedes in Shantung, may be just as good transmittors.

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LANKESTERIA CULICIS A PARASITE OF *AEDES KOREICUS*

In the course of experiments on *Dirofilaria immitis* with *Aedes koreicus*, oöcysts of *Lankesteria culicis* were encountered in several cases in dissecting this species of mosquito. Accordingly, twenty-five larvae and sixteen pupae of this species of mosquito were taken from the pan where they were kept and bred in the laboratory. Dissection of these larvae and pupae showed all positive for gregarines in the mid-gut, and in some of the pupae, oöcysts were found in the malpighian tubes in addition to the gregarines in the mid-gut. Larvae of *Culex pipiens* were also kept in the same pan and on dissecting forty of them, all proved to be negative.

On October 3rd I went to the park where these larvae had been captured, and collected larvae and pupae of *Aedes koreicus* from different kongs. In the laboratory, nineteen mosquitos hatched out, only one of which was found positive on dissection. Seven larvae were dissected, all of which were negative. Twenty-six pupae were dissected, seven being positive. Unfortunately the season was too advanced to get more specimens for dissection. However, it is apparent that the larvae kept in the laboratory on dissection were all found infected, whereas of those newly-collected from the park, only comparatively few were infected. This is probably, as noted by Wenyon (1926), due to the fact that the mosquitos when hatched out and left in the cage for a day soon defecate so that the mature oöcysts pass into the pan and all the larvae in the pan become infected, whereas in natural breeding places in the park only certain kongs were infected.

In larvae of *Aedes koreicus* gregarines were found present in the mid-gut attached to the epithelium. These gregarines vary in size,

EXPLANATION OF PLATE XI

Fig. 1-A. Section of the head and proboscis of *Aedes koreicus* 13 days after feeding on dog infected with *Dirofilaria immitis* showing mature larvae in the head and labium.   × 45.

   1-B. Section of the head and proboscis of *Anopheles hyrcanus* var. *sinensis* 15 days after feeding on dog infected with *Dirofilaria immitis* showing 3 mature larvae in labium and larvae in cross section in lower part of the head. × 50.

Fig. 2. Section of *Aedes koreicus* 15 hours after feeding on dog, showing *D. immitis* larvae in the lumen and cells of the malpighian tubes.   × 270.

Fig. 3. Dissection of *Aedes koreicus* 6 days after feeding, showing both living and calcified larvae of *A. koreicus* in malpighian tubes.   (Calcified larvae dark in lower part of the photograph, living ones in the upper part.)   × 60.

Fig. 4. Section of *Aedes koreicus* 15 days after feeding with calcifying mature larvae of *D. immitis* in malpighian tubes. (Two larvae in the left part of the photograph.)   × 70.

Fig. 5. A mature larva in malpighian tube of *Aedes koreicus* 12 days after feeding.   × 100.

Fig. 6. Dissection of *Aedes koreicus* 9 days after feeding, showing larvae of *D. immitis* in malpighian tubes.   × 75.
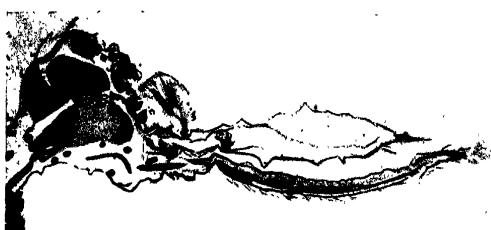
Fig. 1-A

Fig. 1-B

Fig. 4

Fig. 2

Fig. 5

Fig. 3

Fig. 6

EXPLANATION OF PLATE XII

Fig. 1. Longitudinal section of the fourth stage larva of *Aedes koreicus* showing gregarines *Lankesteria culicis* in mid-gut. × 125.

Fig. 2. Dissection of *Aedes koreicus* showing gametocysts and oöcysts of *Lankesteria culicis* in malpighian tubes. (Single oöcysts in the malpighian tubes in upper part of the photograph. Gametocysts in the malpighian tubes in lower part of the photograph and some in the hind gut.) × 90.

FIG. 1

FIG. 2

SPIRILLUM MINUS Carter, 1887, THE AETIOLOGICAL AGENT OF RAT-BITE FEVER : A REVIEW

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PLATE XIII

HISTORICAL

In man rat-bite fever has long been recognised as a definite clinical entity. Early accounts of the disease were given in America by Wilcox (1840), Watson (1840), and Gilliam (1868), in France by Millot-Carpentier (1884), and by Peña y Maya (1885) in Spain. It has been known for a very considerable time in Japan, where the living conditions are such that the rats come into closer contact with human beings and bites are more frequent, but it was not until 1892 that Katsura first gave a clear description of the malady. In 1902 Miyake published an account of the disease under the name ' Rattenbisskrankheit,' and, at the same time, drew attention to the Japanese term ' Sodoku ' (from *so*, a rat, and *doku*, poison), a particularly apt designation. Thereafter numerous attempts were made to isolate the aetiological agent. Probably the first step in the solution of this problem was accomplished when Hata (1912), Surveyor (1913) and Dalal (1914) reported cures with salvarsan having been induced to give this drug a therapeutic trial probably because the periodic fever, the local lesion and the peculiar eruption were suggestive of an infecting organism allied to the spirochaetes. It was left, however, to Futaki and his associates (1916) to make the actual discovery of the causal organism on which they bestowed the name *Spirochaeta morsus muris*.

SYMPTOMATOLOGY

The diagnosis in the majority of instances presents but little difficulty for the clinical signs are usually well-defined and the history of a wound caused by the bite of a rat or other animal is in itself most suggestive. As a rule the bite wound heals cleanly

and until the onset of the disease causes no trouble. There is an incubation period of varying duration, commonly between one and three weeks after the bite, but occasionally as short as three days (Miyake, 1902 ; Hata, 1912; Costa and Troisier, 1918) or four days (Knowles and Das Gupta, 1928), or again it may be prolonged up to five months (Hardy and Savery, 1927). The onset of the fever is sudden and is ushered in by a rigor during which the temperature quickly rises. The pulse is quickened in rate and is softer and the respirations are also increased. There may be headache of varying degrees of severity, and sometimes, in the graver cases, delirium and prostration. Pain in the joints, bones and muscles, especially of the legs and arms, is of common occurrence and may or may not come on coincident with the first febrile attack, but if not will most probably transpire during the second or subsequent pyrexial periods. The rigor which precedes the attack of fever may occur without prodromal signs or there may be preliminary headache, malaise, giddiness, and less frequently vomiting.

The Primary Lesion. The wound caused by the bite heals cleanly unless there has been superimposed septic infection. Under certain circumstances this superimposition of a septic condition may be mistaken for or may mask a true rat-bite fever (Knowles and Das Gupta, 1928). In such there is suppuration at the wound site, a marked polymorphonuclear leucocytosis and the temperature chart is entirely different, while the chances of finding *S. minus* in the exudate are practically negatived. About the period when febrile symptoms become manifest there is pain in the scar and the neighbouring tissues become swollen and oedematous. A proportion of cases eventually go on to ulceration, the ulcer presenting a clean surface, indurated edges and a serous discharge (often containing *S. minus*), and resembling the scrotal lesions produced experimentally in guinea-pigs (McDermott, 1928). This ulcer has been stated by Arkin (1920) and by Adams (1925) to resemble closely an extra-genital chancre. There is inflammation in the regional lymphatics and the glands are swollen, firm in consistence but non-adherent. The intensity of the local condition differs markedly in degree but it is rare, although it has been recorded (by Row, 1912 ; Lagriffe and Loup, 1917 and others), not to find a primary lesion and

adenitis. There is a recrudescence of the pain and local inflammation with each succeeding bout of fever ; in favourable cases the wound eventually heals leaving a scar of a bluish or purplish tint.
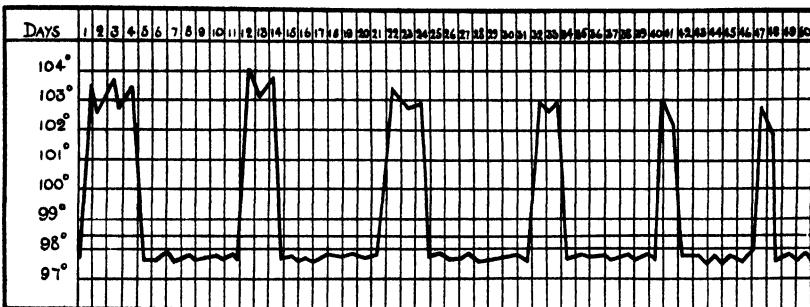
CHART OF THE TEMPERATURE IN RAT-BITE FEVER.
After Knowles and Das Gupta (1928) and other authors.

The Fever. The fever varies in intensity according to the severity of the infection and the temperature often may reach 104° F., although in other cases it may never exceed 101° F. to 102° F. The pyrexial period usually lasts from two to three days, after which the fall to normal is as sudden as the onset. There then follows an interval of varying duration of about three to seven days, which is abruptly terminated by another sharp rise sometimes accompanied by a rigor. The number of the febrile attacks varies in every individual case and is dependent to a large extent on the virulence of the infecting strain of the parasite and the degree of resistance exhibited by the host. It is probable that the majority of infections, even if untreated, would gradually subside, each subsequent attack of fever being less severe than the preceding, until the patient returned to a more or less normal state of health, but there are cases on record, such as those of Corinaldesi (1924), Surveyor (1913) and van Lookeren-Campagne, in which symptoms were prolonged over periods of four, eight and twenty years respectively. During the remissions all the symptoms, the pains, headache, and giddiness, usually disappear.

The Rash. As a general rule the rash, which is most characteristic, is present only during the pyrexial periods and subsides with the temperature ; it can be made to return, in certain instances, if the patient is placed in a hot bath. Its appearance is synchronous with

the onset of fever, not necessarily with the first attack, and may take place on any part of the body, face, neck, chest or widely spread all over the body and sometimes even affecting the mucous membranes (Surveyor, 1913; Zannini, 1914; Collier, 1924; Giglioli, 1927). The most marked feature about the eruption is the colour, which is of a peculiar purplish shade contrasting fairly sharply with the surrounding tissues. It is maculo-papular, occurs in slightly raised areas of varying size, is painless and free from itch, and will disappear only when considerable pressure is exerted. Slight variations, such as ecchymosis (Vorpahl, 1921), may occur in the nature of the rash, but they are unusual. With the sudden fall in the temperature the rash fades and gradually disappears, while very infrequently it is followed by a certain amount of desquamation.

Other Symptoms. In addition to the three cardinal symptoms—the recurring inflammation at the bite and in the regional lymph system, the remittent nature of the fever and the characteristic rash—numerous other clinical manifestations have been described, but it seems probable that a large number of these are more or less accidental or are due to intercurrent causes. Mention must be made, however, of an urticarial rash, which may be observed in the terminal stages of the disease (Miyake, 1902, and d'Halluin and Fievez, 1918), and has been regarded, from the point of view of prognosis, as a hopeful sign. As would be expected in such a febrile disease, there may be, especially in cases of long duration, a slight secondary anaemia, and, as regards the gastro-intestinal tract, constipation and some signs of gastro-enteritis (Millot-Carpentier, 1884; Peña y Maya, 1885; Cook, 1886 and Annibale, 1920). Diarrhoea is seldom present, but when it does occur is probably associated with a catarrhal condition of the intestinal mucosa (Schockaert, 1928) such as was described at post-mortem examinations by Kaneko and Okuda (1917).

There is no liver enlargement, an important diagnostic point in the comparison of this disease with other spirochaetoses such as acute infective jaundice (Weil's disease), and there is no jaundice. The kidneys show signs of toxic irritation, albumin and more rarely casts appearing in the urine (Miyake, 1902; Blake, 1916; O'Carrol, 1912). The heart and lungs are scarcely affected; in the

case of the former cardiac failure due to myocarditis, according to Kaneko and Okuda (1917), may be the cause of death in severe infections, while the latter may show some slight bronchitis, broncho-pneumonia (Schockaert, 1928), pleural exudate (Middleton, 1910) or even a double-sided purulent pleurisy (O'Carrol, 1912). Symptoms referable to the nervous system are present in nearly all instances. Headache, giddiness, tinnitus, are all common during the bouts of fever ; less frequently there may be delirium or coma. The muscle pains are very common in the extremities and are probably most marked in the calf of the leg. As noted by Gerbasi (1927), by Lange and Wolff (1921), and by Mauriac (1918), pains around the joints are not infrequent. Anaesthetic patches may occur usually on the lower extremities and hyperaesthetic areas may be found on any part of the body. Photophobia (Schockaert, 1928) and con-junctivitis (Frugoni, 1912) have been noted.

The Blood. With regard to the blood it is generally agreed that there is a degree of anaemia proportional to the severity and duration of the disease, and, in long-standing cases, giving counts of the red blood corpuscles of as low as 1,390,000 (O'Carrol, 1912). Each febrile attack is associated with erythrocyte destruction and sub-sequent appearance of urobilin in the urine (Schockaert, 1928), while during the quiescent phases there are attempts at regeneration evidenced in blood films by anisocytosis, polychromasia and the presence of normoblasts. A moderate leucocytosis (12,000-20,000) may be encountered at the onset of the fever ; this is maintained during the attack but, later, as the fever subsides, it gives place to a lymphocytosis. The eosinophiles are diminished in number and it would appear (according to Schockaert) that the eosinophilia noted by Frugoni (1912), Walch (1923), Bergamini (1915) and other observers only appeared between the attacks and towards the end of the infection when it may be regarded as a hopeful sign. Kuipers and Ruys (1929) made a very thorough investigation of the leucocyte content in a case under their observation. Leucocyte counts were performed daily over a period of about four months' duration. During the actual attacks there was a leucocytosis with a relative lymphopenia and aneosinophilia. There was a rise in the number of eosinophiles a few days before the attacks and during convalescence there was a definite eosinophilia. It was found that even after the

disease had apparently subsided these alterations in the blood picture occurred regularly for some time and the authors suggest that after seeming recovery from rat-bite fever attacks still occur which remain under the clinical threshold.

Serology. The majority of cases exhibit a positive Wasserman reaction (Blum and Clement, 1925 quote a figure of about 50 per cent. positives). Recently Nakamura (1928) has succeeded in obtaining a complement fixation test for rat-bite fever. He prepared an antigen of (infected ?) guinea-pig's heart, 1 gram extracted with 5 c.c. of alcohol. This was diluted with three times its volume of alcohol and to each 5 c.c. of the resulting liquid was added 0.3 c.c. of a 1 per cent. alcoholic extract of cholestrol. The preparation was again diluted with six times its volume of saline solution. The diazo-reaction was stated to be positive by Toriyama (1917) and v. Lookeren-Campagne and negative by Gerbasi (1927).

Mortality. According to Miyake (1902) the case mortality in untreated cases is about 10.5 per cent., but since the introduction of specific treatment with arsenical preparations, noticeably salvarsan, this figure has been very much reduced, in Japan possibly some 2 per cent. and elsewhere probably less than that percentage.

AETIOLOGY

The publication of Miyake's paper in 1902 served to focus attention on the condition with the result that considerable research was carried out and attempts were made to isolate the etiological agent. Shikami (1907) attributed rat-bite fever to a member of the Telosporidia ; Middleton (1910) thought that the causative organism might be a *Diplococcus ;* Ogata (1911), who allowed rats to bite guinea-pigs and thus experimentally transmitted the disease, considered that it was due to an *Aspergillus ;* Proescher (1911) described *Bacilli (B. septico-muris)* which were very numerous in the base of the wound. More importantly Schottmüller (1914) isolated from two cases, one resulting from the bite of a rat, the other from that of a South African squirrel, two species of *Streptothrix*, which were named respectively *S. muris ratti* and *S. taraxeri-capapi.* Blake (1916) was able to demonstrate a similar *Streptothrix* in the

vegetations on the heart valves in an endocarditis following on the bite of a rat. The serum of the patient strongly agglutinated the organism in question. In this connexion the writer (1924), reviewing the literature on the subject, drew attention to the fact that Tunnicliff (1916) had recorded the finding of a *Streptothrix* in rats with broncho-pneumonia, and, especially as Tunnicliff and Mayer (1918) had noted a further case in man of *Streptothrix* disease, concluded that it was possible that at least two generalised diseases might be transmitted to man through the bites of rats and associated animals, the true rat-bite fever and the *Streptothrix* infection probably causing endocarditis. Knowles and Das Gupta (1928), however, are clearly of the opinion that such *Streptothrix* infections are of a secondary nature, and in one case they described it undoubtedly was so, but McDermott (1928) noted that in such cases there is almost always suppuration at the site of the bite, whereas in *S. minus* infections suppuration does not take place.

The discovery of *Spirillum minus* in man was made almost simultaneously by two groups of workers in Japan. Futaki, Takaki, Taniguchi and Osumi (1916) demonstrated the organisms first in fluid obtained by gland puncture from one case and secondly in a piece of skin excised from one of the rash papules. They named it *Spirochaeta morsus muris* and considered it to be of the nature of a *Treponema*. The movements of *S. morsus muris* were stated to be very rapid, resembling those of a vibrio. It stained readily with Giemsa's stain. Animal experiments were successful ; monkeys, mice, house rats and white rats were inoculated with positive results. Attempts were made at cultivation and success was claimed on Shimamine's medium, but the culture organisms, as described and figured, differed very considerably from the forms found in man and animals. In cultures forms appeared up to 19μ in length—as against the 2μ or 5μ, or including flagella 6μ to 10μ quoted by these authors for the forms in the host—and having at times as many as nineteen coils. The pitch of the spiral or wave was about 2μ, whereas the blood forms had one coil in 1μ. Futaki and his associates were of the opinion that the organism differed in certain respects from those previously described as occurring in the blood of mice, rats and other animals (*vide infra*), and further that, as no connexion between these organisms and rat-bite fever in man had been demon-

strated, they were justified in considering that the parasite was a new species.

A little later Ishiwara, Ohtawara and Tamura (1917) reported it in the cortex and parenchyma of the supra-renal glands of guinea-pigs inoculated with material from human cases. They also transmitted the disease experimentally by allowing infected rats to bite guinea-pigs (thus confirming the work of Ogata, 1911) and studied the course of the disease in these animals. Row (1917) isolated a spiral organism from a case of human rat-bite fever which was smaller in size than that of the Japanese workers and had fewer coils. On this morphological basis and because he was unable to demonstrate terminal flagella, Row concluded that it was a distinct species and, later, in 1922, he named it *Spirochaeta petit*. Parmanand (1923), however, succeeded in staining the flagella and expressed the view that the organisms in Bombay were similar to those described by Futaki. In 1922 Manson-Bahr, in ' Manson's Tropical Diseases,' referred the causal organism of rat-bite fever to the genus *Leptospira* on grounds which were not stated, while Sangiorgi (1922) suggested the generic name *Treponemella*.

Probably the earliest account of the finding of such a *Spirillum* in the blood of animals is that of Vandyke Carter (1887). This was present in the blood of a rat, *Mus decumanus*, and attention was first drawn to its presence by a quick, twirling movement of the red blood corpuscles in fresh blood. Carter described his organism as an ' extended and uniformly slender filament of clearly spiral construction, having a length commonly somewhat less than, the diameter of a blood disc, but varying from 5μ to 9μ . . . and according to its length presenting from four to eight close spiral turns.' He was unable to make out the presence of flagella at the ends. The movement of the organism was very active, and consisted of ' rotation round the long axis, propulsion either forward or backward, and occasionally an energetic twisting or lashing.' From its morphology, movements and behaviour to reagents Carter concluded that the organism in question was ' a bacterium belonging to the genus spirochaete . . . such as from its small dimensions might be named provisionally *Spirillum minor*.'

Thereafter there occur in the literature numerous mentions of similar organisms, which were found in the blood of animals.

Lingard (1899) found in the blood of a bandicoot (*Mus giganteus*) a small *Spirillum*, which he was able to inoculate into rabbits and guinea-pigs, but in the latter it was possible only to demonstrate the organism in the final stages of the infection. While Lingard's description of the parasite is lacking in detail, · its behaviour in inoculated animals was similar to that of the virus of rat-bite fever as reported by various workers. Borrel (1905) found a spirochaete in cancer growths of mice. This work was confirmed by Calkins and Clowes (1905), Deetjen (1906), Tyzzer (1906-07) and Gaylord (1907).

Wenyon (1906) described a spirochaete in the blood of mice which he named *Spirochaeta muris*. This he considered to be identical with the spirochaete found by Borrel, but with reference to the organism of Carter he states that ' since the morphological characters of spirochaetes are not sufficient to establish the identity of any form it is necessary to rely on other characters, notably their behaviour in various hosts, and their pathogenic or other action. As nothing is known of Carter's *Spirillum* of the rat apart from its morphology, the spirochaete of the mouse must be considered to be new to science.' Morphologically, Wenyon's *Spirochaete muris* closely resembled Carter's *Spirillum minus* and there is now little doubt but that they are identical. Breinl and Kinghorn (1906) isolated a spirochaete from the same source as Wenyon. They considered it to be smaller in size than the one described by Carter and therefore a distinct species for which they proposed the name *Spirochaeta laverani*.

MacNeal (1907) demonstrated the presence of flagella at the ends of the organism in a strain isolated from one out of thirty-nine rats (*Mus decumanus*) caught at Morgantown. He was of the opinion that this parasite was of the same species as those of Carter, Wenyon, Breinl and Kinghorn and Nicolle and Compte (in a bat). Despite the fact that he thought his organism to be the same as that of Carter, and that terminal flagella had been seen, MacNeal adopted Wenyon's name *Spirochaeta muris* var. virginiana.

Zuelzer (1921) suggested that the morphological characters were such as to indicate a closer affinity of the rat-bite fever parasite to the *Spirilla* than to the Spirochaetes. Later Robertson (1924) from a study of the morphology of the causal organism, its behaviour

in inoculated animals and the various accounts in the literature, came to the conclusion that Zuelzer's suggestion was correct, and, further, there was at that time no evidence to suggest that the forms occurring as natural infections in the blood of animals (*Spirillum minus* Carter, 1887 ; *Spirochaeta muris* Wenyon, 1905 ; *Spirochaeta laverani* Breinl and Kinghorn, 1905 ; *Spirochaeta muris* var. *virginiana* ; etc.) differed in any respect from those causing disease in man. This, however, was not in agreement with the view of Kasai (1921), who thought that it was possible to distinguish between two strains, one the rat-bite fever variety (*morsus muris*) and the other the forms found in mice (*muris*). Kasai's hypothesis was supported by Ruys (1925). Four strains of organisms, one from a human case, two from rats and the fourth from white mice, were compared by this observer in their animal reactions. The human and rat strains proved to be pathogenic for monkeys and guinea-pigs while the inoculated animals did not seem to be susceptible to the mouse strain. Serologically the same differentiation appeared to be present. The opposite was expressed by Worms (1926) as a result of the study of a rat strain in comparison with a strain derived from field mice. The findings left no doubt as to the close relationship of the two strains, that of the mouse eventually becoming extremely virulent to guinea-pigs and giving a mortality up to 30 per cent.

It is obvious, therefore, that human strains differ from each other in their infectivity for and reactions in experimental animals. Thus one strain may be highly virulent in guinea-pigs, and show parasites in the animal's blood, whereas another may cause some temporary discomfort only. One strain may be easily infective for rats and another may not. Such differences as do occur between human and animal strains in their infectivity and reactions are no greater than may be found in two divergent human strains. The final proof that *Spirillum minus* is the aetiological agent of rat-bite fever or sodoku and of the co-identity of the rat and mouse strains of the virus is found in the work of Theiler *et al.* (1926) and of Schockaert (1928), both of whom have successfully inoculated man (cases of general paralysis of the insane) with the human strain in laboratory animals and, in the case of the second worker, also with a strain from white mice.

GEOGRAPHICAL DISTRIBUTION

It would appear that the disease is almost world-wide in its distribution, cases having been recorded in nearly every country. Figures have been given by various observers showing the percentage of natural infections occurring among rats, but such observations are of little value as an indication of the frequency with which the disease occurs in man. There is to be considered, firstly, the fact that adult rats, even when experimentally inoculated, often do not have parasites, at least in demonstrable numbers, in the circulating blood, and, secondly, the incidence of the disease is not dependent so much on this factor (the percentage of natural infections) as on the exposure of man to the bites of animals.

ANIMAL VECTORS AND MODE OF INFECTION OF MAN

The majority of cases in man result from the bites of rats, although a certain number may follow on those of other animals such as cats, ferrets, dogs, etc. The scratch of a cat has been incriminated on two occasions (Yamado, 1917, and Sano, 1917), but it is more than likely that these are merely examples of direct or mechanical contamination with infected material as are also the cases described by Atkinson (1913) from a kitten and a ferret both of which had been in contact immediately previous to the bite with dead rats. Iyer (1929) recorded a case, diagnosed on clinical grounds, in a patient bitten by a bandicoot. This occurred in Madras. Smallwood (1929) also reported a case, the diagnosis of which was dependent on the clinical signs, following the bite of a young pig.

The question of how the *Spirillum* is actually transmitted from the rat to man is one which has led to considerable discussion, for the organism cannot be demonstrated in the saliva of the animal and experimental inoculations with that secretion have been also, with one exception, negative. The exception, which was probably more in the nature of an accident, was that of Kusama, Kobayashi and Kasai (1919), who managed to infect one mouse by this means. Up till recently it was thought that, since the saliva as a rule was non-infective, the small abrasions in the gums and buccal mucosa resulting from the act of biting, might cause small haemorrhages and that the blood containing *Spirilla* could thus contaminate the

bite. Mooser (1924), however, suggested an alternative mechanism. He drew attention to the fact that rats, some time after infection, showed certain eye symptoms such as conjunctivitis, keratitis, iritis and palpebral oedema, and that in most instances the accompanying secretion was infective even though the blood was non-infective. He therefore concluded that the rats, even in the absence of injuries to the mucous membrane of the mouth, might be infective by means of this optical secretion, the *Spirilla* reaching the mouth through the lacrymo-nasal duct. As noted by Mooser and also by McDermott (1928), desquamated duct cells and erythrocytes may occur in the lumina of the salivary gland ducts, a fact which renders it possible for the organism to be present in the saliva in the later stages of the disease, and, further, McDermott also points out that in rats, late in the infection, ' tertiary ' lesions (*vide infra*) are of almost constant occurrence. These lesions frequently ulcerate—thus discharging *Spirilla*—into the bronchi, mouth and upper alimentary tract. Such records as are available of the rats which have been known actually to transmit the disease to man would suggest that the infecting animals were in poor physical condition similar to that seen in the so-called tertiary stage noted above. Honda (1928) has carried out a series of experiments (with *Treponema recurrentis* as well as *Spirillum minus*) with the object of determining whether the organisms could pass through undamaged skin and mucosa. *S. minus* did not produce infections when applied to the skin and only about a quarter of the cases were positive where the application was made on the buccal mucosa or the conjunctiva.

MORPHOLOGY

Material. *Spirillum minus* is best studied in fresh preparations under dark ground illumination for, no matter what staining or fixing technique may be adopted, distortions, in such a small organism disproportionately important, are bound to occur, giving a false impression both of size and structure. Further, the terminal flagella do not stain easily even when lying free from the body. Material for study is not easy to obtain from man. *Spirilla* may be present in various situations, noticeably the serous exudate from the primary lesion, but as a rule they are too scanty to permit of accurate

examination or detailed observations. In the mouse, on the other hand, the parasites are often quite numerous, two, three, four or even more occurring at times in each field of the microscope of the peripheral blood. An even better medium than the blood of the sub-inoculated animal is the peritoneal fluid in which large numbers are present during the greater part of the infection. Why this should be the case is not quite clear ; it is possible that the peritoneum is a suitable nidus for their growth and multiplication or it might be to a certain extent the result of the inoculation which is commonly made in this situation. There is one possible disadvantage to the use of this material which is that the majority of the forms encountered seem to be of the longer variety while short forms, $1 \cdot 5\mu$ to 4μ, are comparatively scanty.

Technique. For the demonstration of the flagella the best method, apart from dark ground illumination, would appear to be Tribondeau's modification of Fontana's silver nitrate stain as recommended by Schockaert (1928) and by Knowles and Das Gupta (1928). Although this modification requires the use of methyl alcohol as a fixative and is otherwise very similar to the original technique of Fontana it does not cause the flagella to appear as thick and single, a disadvantage stated by McDermott (1928) to occur in Giemsa stained preparations fixed by methyl alcohol and also in Fontana stained films. The Tribondeau-Fontana method (after Knowles and Das Gupta, 1928) is as follows :—

(1) Make a thin film of the suspected material. Dry in air.
(2) Fix with Ruge's solution for one minute.

Glacial acetic acid	... ...	1 c.c.
Formalin (40 per cent.)	...	2 c.c.
Distilled water ...	... ...	100 c.c.

(3) Drain the fixative off the slide and flood with methyl alcohol. Complete the fixation by flaming off the methyl alcohol.

(4) Mordant for one minute, warming gently until steam rises in

Fresh tannic acid	... ...	5 grams
Distilled water ...	... ...	100 c.c.

(5) Wash very thoroughly in distilled water.

(6) Stain for one minute, warming until steam rises, in Fontana's silver nitrate solution made up as follows :—To a 5 per cent. aqueous solution of silver nitrate Liq. ammoniae is added

drop by drop until the precipitate which forms redissolves in excess of ammonia. With a fine pipette more silver nitrate is added until the precipitate just returns and the solution, after shaking, is opalescent.

(7) Wash in water, allow to dry, and, if desired, mount in Canada balsam.

If more intense staining is required, the various steps from No. 4 onwards may be repeated.

The method recommended by Adachi (1921) and confirmed by McDermott (1928) consists of (1) fixation for from 30 to 60 seconds over a solution, osmic acid 1 gram, mercuric chloride (corrosive sublimate) 5 per cent. 10 drops, distilled water 100 c.c. and (2) staining for about twelve hours in Giemsa's solution (1 part of stain to 10 parts distilled water) to every 10 c.c. of which 0·6 c.c. of a 1 per cent. aqueous solution of potassium carbonate has been added.

When the organisms are fresh the movement is so rapid that the details of structure cannot be seen ; for observations under the best conditions Schockaert recommends the examination of a mixture of infected blood in which the serum has only faintly lytic properties. Under such circumstances this author states that at the moment of admixture the motility of the *Spirilla* is slowed and the flagella lose their oscillatory movements. At room temperature, if precautions are taken to avoid drying, movement will persist for more than twenty-four hours. Bright light is said (Futaki *et al.*, Adachi, McDermott) to inactivate the parasites so that observations on a single living specimen can only be carried out for a few seconds after which it ceases to move, swells and loses its waves. This, however, is denied by Schockaert, who found that exposure to the light passing through the microscope did not seem to modify the movements in any way. The writer's experience, using a bright light (30 or 100 candle power point-o-lite) is that the movements certainly are slowed but the effect is not nearly so rapid as indicated ; active movements may persist for an hour or longer and the structure during that time does not alter appreciably. Using fresh films from the peritoneal fluid, exposure to cold, such as is easily obtained by placing the slide on a block of ice for a few minutes, is a useful expedient, for some of the individuals retain their original motility while others are inactivated or merely slowed. The flagella, their

movements and disposition can be seen clearly and, although a measure of swelling may take place, the characters of the body are still quite recognisable.

The Body. A certain amount of doubt is still present as regards the structure of the body. Ruys (1926) and McDermott (1928) state that it does not have a spiral formation for, under dark ground illumination, the waves can be seen lying in one plane. Other observers have described it as forming a true spiral ; it is certainly the case that sometimes the waves (or curves) do not appear to be so evident, but, on the other hand, it is equally difficult to make sure that this is not due to a general thickening and straightening of the body. With reference to the flagella, McDermott states that they are ' long and wavy, frequently continuing the direction of the body waves for one or two turns, but gradually becoming almost straight.' The flagella, when examined under dark ground illumination, are in nearly every instance in spiral form, and, if it is the case that they follow the curves of the body, this would suggest a coiled shape for the body.

A marked feature of the body is the relative rigidity. Occasionally, as a result of extraneous influences, bending may occur, but as soon as the causal factors are removed it returns to the original state. There is no undulatory motion ; the rat-bite fever organism retains its fixed shape during movement. Forms which are in process of division are frequently bent, but such individuals are usually easy of recognition for they have two more or less equal parts joining at an angle, not a single mass curved in the centre. The rigid character of the body serves as a point of differentiation between this *Spirillum* and the members of the genus *Treponema*. With regard to the size of the parasite considerable variations have been noted. Thus it varies in different animals, and also in the same animal from day to day. There appears to be a certain tendency (Robertson, 1924) to uniformity of size in any given animal on any given day, sometimes long forms preponderate, at others shorter or intermediate varieties are more in evidence. The greatest body length is some 9μ or 10μ and the shortest $1\cdot5\mu$. The average size probably lies between 3μ and $5\cdot5\mu$. Thickness is much more difficult to estimate ; Robertson (1924) estimated it at about $0\cdot2\mu$ and Schockaert (1928) at $0\cdot1\mu$. The width of the

organism as a whole, i.e. of the spiral, is about $0\cdot7\mu$. The waves or spirals, measured from crest to crest, each occupy from $0\cdot8\mu$ to 1μ and are very regular and fairly sharp. The number of such waves or coils in each *Spirillum* is accordingly directly proportional to the length, varying from one and a half to eight or nine. The ends tend to be blunt and rounded in the majority but in some there is a tendency to a more tapering extremity. In such as have the sharply-defined, blunter ends the terminal flagella are more commonly multiple whereas the more pointed types seldom have more than a single flagellum.

The Flagella. Flagella are present at both ends of the body except, possibly as a result of division, in some of the shorter forms which are clothed at one end only. There is no relation between the number of flagella at one end and those at the other, nor between the number of flagella and the size of the body (McDermott). At each end they vary from one to seven, although Schockaert maintains that the flagellum is usually single. It should be noted that in dark ground preparations in which, either through exposure to light, cold, or some other mechanism, movement has been slowed, or in stained films, the flagella may appear to be single through several winding together. Occasionally several flagella may take origin from a common stalk. Dividing forms often have one or more flagella at the zone of separation. In active movement the flagella are extremely difficult to make out ; those at the anterior end trail backwards alongside the body while the others project posteriorly. In fixed and stained smears they are far from easy to demonstrate because, for some reason, they do not stain readily, even using the same technique constant results cannot be obtained, and they so frequently wrap themselves round the body. The flagella may measure up to 7μ or 8μ.

Multiplication. Multiplication takes place by transverse binary fission into two more or less equal portions. A constriction appears about the middle of the body and this gradually deepens until the two parts are connected by the merest thread, which eventually breaks as the daughter individuals draw apart. Certain peculiarities of movement may be seen in these dividing forms owing to the different alignment of the two halves. Under circumstances where multiplication is, as it were, unusually rapid, e.g. in the peritoneal fluid,

division of the body into three, and more rarely four, is sometimes observed.

Movement. Movement is most characteristic. It is also very rapid and consists chiefly of progression in a straight line, with sudden dashes to and fro, or in any direction, when either end for the time being may be the anterior. The lashing movements of the flagella frequently impart a spinning motion to the body as a whole. So far as is known undulatory movements do not take place although this is a point which again is difficult to resolve because the impetus given by the flagella may produce a slight movement of the body closely simulating undulation. This is especially the case when the movement is very slow or indeed has almost stopped and there is only a gentle rise and fall in a vertical direction, slight variations taking place in the focus both of the microscope and, more importantly, of the light from the dark ground condenser.

NOMENCLATURE

The question of structure is an important one, for the organism cannot be classified correctly until the details are accurately established. Leaving out of account for the time being the debatable point as to whether the morphologically identical forms found naturally in certain animals are specifically the same as the virus of rat-bite fever, it is convenient to consider here the systematic position of the parasite. Futaki and his associates placed it originally in the genus *Spirochaeta*, and Futaki (1926) still calls it a ' Spirochaete.' In 1924 Robertson concluded that it was a *Spirillum*, but Mooser (1924, 1925) classified it with the *Treponemata*. It has also been placed (Manson-Bahr, 1925) in the genus *Leptospira*, but as no reasons were given for this procedure and as there is not the slightest similarity between its structure and that of any *Leptospira* this can quite well be disregarded. With regard to the two genera *Spirochaeta* and *Treponema* the issue is further obscured by the fact that the classification of the ' spirochaete ' group is admittedly unsatisfactory and inadequate, with the result that some authors apply the generic name *Spirochaeta* to all such spiral organisms. For present purposes, without expressing any view on this vexed question, it will be assumed that they refer to distinct genera,

following in this respect Dobell (1918), who pointed out that the type species of the genus *Treponema* (*T. pallidum*) was morphologically so different from *Spirochaeta plicatilis* (also the type species) that they could not be considered as belonging to the same genus. The rat-bite fever organism with its non-flexible body, multiple terminal flagella and absence of an axial filament, is much more divergent in character from the *Spirochaeta* than are the *Treponemata*, and the possibility of inclusion in that genus may therefore be ruled out. Much the same reasons hold good for exclusion from the genus *Treponema* within which it was placed by Mooser (1924). Bending of the body, such as was described by this worker, does not occur except as the result of extraneous influences or in dividing forms and whereas in a true spirochaete (i.e. *Treponema*) the coils are formed by the bending of the organism as it travels, the coils (or waves) in *Spirillum minus* are preformed (Knowles and Das Gupta) and the organism has a rigid structure. It may be concluded, therefore, that it is distinct from the various genera of Spirochaetes.

Zuelzer (1921) suggested that it should be regarded as a *Spirillum* and Robertson (1924) definitely referred it to that genus. With a few exceptions this has been generally accepted, but attention must be drawn to the possibility that, if the waves of the body lie in one plane, as was stated to be the case by Ruys (1926) and McDermott (1928), and there is no true spiral structure, it may have to be classified not as a *Spirillum* but in some other genus.

CULTIVATION

Futaki and Colleagues (1917) claimed to have cultivated the parasite on Shimamine's medium, but, as noted by Robertson (1924) the culture organisms differed very considerably from the forms found in man and animals, so much so, indeed, that it is possible some other spiral organism had been present in the cultures as a contamination, or confusion had arisen with the threads of fibrin (the ' pseudo-spirochaetes ' of Thomson, 1923) which are almost constantly to be found in a medium of that nature. In 1925, Joekes stated that he had been successful in attempts at cultivation. His medium consisted of an ' inspissated horse-serum slope (as commonly used for the cultivation of the Klebs-Löffler bacillus) covered by 10 c.cm.

of Vervoort's medium with a pH of 7·2.' Vervoort's medium as used by Joekes was composed of 1 g. (? gram) peptone and 3 c.cm. normal phosphoric acid dissolved in 900 c.cm. distilled water. The pH of Vervoort's medium, originally 6·6, should not be re-adjusted before adding to the serum slope. According to Joekes *Spirilla* were found alive and actively motile after three weeks whether incubated at room temperature or at 37° C. Cultures were not obtained from mice, only from guinea-pigs.

A most curious feature of Joekes' cultures was that they contained a motile coliform bacillus as well as the *Spirilla*. No one, so far, has been able to repeat these cultivation experiments, although many workers have tried to do so. McDermott, Schockaert, and Knowles and Das Gupta all record failures. Commenting on this subject Knowles and Das Gupta stated that they had attempted repeatedly to cultivate this parasite but without success.

EXPERIMENTAL INOCULATION OF ANIMALS

Before proceeding to the consideration of the inoculation experiments performed on various animals and the reactions of these experimental hosts to the virus, it is expedient to mention briefly the attempts which have been made to associate insects with the dissemination of the disease, not in the case of man but in naturally infected animals. The majority of such observations have been made on the rat flea and probably the most direct evidence, in a negative sense however, is that of Basu (quoted by Knowles and Das Gupta, 1928) proving that although the *Spirilla* could survive for twenty-four hours or longer in the gut of fleas fed on infected mice or guinea-pigs, there was nothing to indicate that they ever invaded the coelomic cavity or the salivary glands. This experiment is suggestive in that positive results might be expected if the gut contents of insects from infected animals were injected into fresh hosts. Schockaert, however, having ground up and injected into mice lice from two heavily infected guinea-pigs, did not succeed in producing infection corroborating in this respect the work of Wenyon (1905). It may be concluded, therefore, that there is no evidence in favour of insect transmission.

Infection may be produced by the eating of tissues from infected animals, but the mechanism in such instances would appear to differ

only slightly from the usual for, unless abrasions or injuries are present in the upper part of the alimentary tract thus allowing ingress to the *Spirilla,* infection will not supervene. Schockaert, for example, fed six mice on tissues from guinea-pigs without producing a single infection although Kusama recorded a small percentage of positives after a similar infected meal. Mooser (1925) noted an instance in which transmission was probably ·effected during coitus. A male rabbit developed a chancre on the prepuce within fifteen days after coitus with an infected doe showing some oedema of the vulva and a little later developed a typical generalised infection.

Mouse. In the list of susceptible animals may be included mice, white rats, guinea-pigs, rabbits, cats, ferrets, and *Macacus rhesus.* Mice are easily the most suceptible of all, followed in turn by young guinea-pigs and by adult guinea-pigs. The ease with which infection can be produced in mice by inoculation, the convenience of maintaining any given strain by subinoculation from mouse to mouse, and the comparatively constant course exhibited by the disease in that animal render it the most valuable experimental host. Mice as hosts have one possible disadvantage, viz. :—the frequency with which natural infections may be found. It is advisable, therefore, before inoculations are performed, to control the experiments by careful examinations of the peripheral blood for some time previous to the actual injection. On four occasions since 1924 the writer has discovered spontaneous infections of *S. minus* in laboratory mice which were either stock animals or were carrying some other parasite (*Trypanosoma cruzi, Treponema recurrentis*).

According to Knowles and Das Gupta, inoculation of mice with blood from suspected human cases forms the most reliable and the most certain method of diagnosis. The blood must be obtained while the patient is in one of the pyrexial periods and they recommend the withdrawal from a vein of 3 c.c. blood which is immediately inoculated intraperitoneally into a guinea-pig (2 c.c.) and into a mouse (0·5 c.c.). In this manner, out of twenty-seven cases in man, only three gave negative results after injection into animals. As regards the actual injection it does not appear to matter appreciably whether it is performed intraperitoneally or subcutaneously. The period of incubation varies from three to fifteen days and on the

average lasts from about eight to ten days. The average is not constant for all strains, since some are more virulent than others. On their first appearance in the peripheral blood the organisms are scanty in number but they soon start to increase, so that after another seven days or so they have attained their maximum, when four or five and in severer cases even more (twelve to fifteen) may be counted in each microscopic field. The maximum intensity is prolonged for about eight days and there is then a gradual fall, with the result that after several weeks only a few rare individuals can be found after prolonged search. Occasional relapses do occur, and, as noted by Robertson (1924), it is usually possible to recover the strain by sub-inoculation after a considerable time has elapsed. McDermott states that the infection may be demonstrable by this means up to six and a half months from the date of the initial infection. Robertson also drew attention to the fluctuations which occur in the numbers of the parasites from day to day. Such increases and decreases—sometimes even temporary disappearance—are more noticeable during the time when the parasites are gaining in numbers. In the section on morphology it was mentioned that there is a tendency for a degree of uniformity in size of the organisms to occur on any given day. On some days long, dividing forms are more prevalent while at other times shorter or intermediate forms preponderate. The morphological variations would appear to correspond to a certain extent with the fluctuations in the actual numbers.

Schockaert (1928) in his animal experiments was chiefly engaged in comparing the reactions of two strains one from a case of rat-bite fever in man with a second from mice. He concluded that the mouse as an experimental animal was equally susceptible to both. Infected mice (according to McDermott) do not appear to be otherwise than in perfect health and, even after an infection of prolonged duration, only show post-mortem a slight splenic enlargement. The disease in mice, according to Schockaert, differs but little from that in rats except that the pathological lesions are much less marked. The virulence of various strains towards mice is obviously not a constant factor. The strain described by Robertson (1924), which was isolated from a sodoku patient, at first caused the animals practically no inconvenience but later, after about a year, became

virulent, killing the mice in about fourteen days. This increase in virulence was gradual—concurrent infections were excluded as far as possible—and the rapid mechanical transmission from host to host might serve as an explanation for its occurrence. A second strain discovered accidentally in stock mice by the writer also proved to be definitely pathogenous, for while not fatal to every animal, those which harboured parasites, acquired either naturally or by injection, were certainly very ill. The other strains were of a much lower degree of virulence. No differentiating points could be discerned between human and animal strains.

Salimbeni, Kermorgant and Garcin (1925), while working with the rat-bite fever organism in mice, had their attention drawn to the extreme infectivity of spleen pulp and suspected that a filterable form of the virus might be present in the tissues of that organ. They accordingly filtered spleen pulp diluted with water first through cotton wool and secondly through a Chamberland L 3 filter. The filtrate was injected into mice with positive results. McDermott repeated this experiment but was unable to confirm the presence of filterable forms. Troisier and Clément noted the presence of the specific parasite in the blood of young mice born of infected mothers. Salimbeni and his associates also favoured the view that hereditary transmission does occur, and they instance a case in guinea-pigs in which two young individuals were both found to be infected and died, the one in 31 days and the other in 68 days after birth. *Spirilla* were always present in their blood. Abe (1924), Worms (1926), and McDermott (1928), however, are inclined to doubt the possibility of hereditary transmission. The last of these workers (McDermott) ground up the tissues from a mouse newly-born of an infected mother, inoculated the resulting emulsion into a series of mice but failed in each instance to obtain an infection.

Rat. The disease in rats is of particular interest in that this animal is the one which commonly transmits it to man and also because the course followed by the infection bears a marked similarity to that of syphilis in the human subject. This latter fact was emphasized by McDermott (1928), who gave at the same time a clear account of the various pathological lesions. This observer divides the malady into several stages. There is first a period of incubation followed by a ' primary ' stage during which the organisms

are localised in the lesion and the regional lymphatic system. The presence of a local reaction, however, is denied by Schockaert. The 'secondary' stage may start within anything from four to thirty days after the inoculation and its onset corresponds with the appearance of the *Spirilla* in the peripheral blood. During this phase the numbers of the *Spirilla* increase, attain their maximum which is less than that seen in the blood of mice, and then gradually diminish, finally disappearing in about four weeks time. Four months after the start their blood is no longer infective when injected into mice. Next there follows a latent period, the blood being free from organisms and this merges imperceptibly into the 'tertiary' stage. In this, the tertiary stage, the animals develop keratitis, conjunctivitis and iritis after from $3\frac{1}{2}$-$4\frac{1}{2}$ months (Mooser, 1924). McDermott found that death usually supervened after nine months and post mortem the rats had 'gummatoid' lesions in the lungs, lymph glands (especially those in the mediastinum) and occasionally in the liver and spleen. These lesions began in the lymphoid tissue with hyperplasia and proliferation of the reticulo-endothelial cells and this was followed by necrosis. Eventually in the lung nodules were present of all sizes up to that of a hazel nut and composed of necrotic material surrounded by a zone or capsule of fibrous tissue. That this necrotic area contained *Spirilla* was proved by inoculation experiments into mice. Transmission of the disease to man can occur, and this is probably the commonest method, during the tertiary stage when the organisms from the eye discharge or bronchial secretion contaminate the saliva.

Guinea-pigs. McDermott carried out a careful series of observations on rat-bite fever in sub-inoculated guinea-pigs. In contradistinction to Schockaert he found that a definite local lesion occurred at the site of injection, especially when it was situated on the scrotum or labia majora, with ulceration and a serous discharge containing *Spirilla*. There was enlargement of the local lymph glands.

Considerable variations have been noted in the virulence of various strains to guinea-pigs. Ogata, Ishiwara, Mooser, Ruys and Worms all found that the human strains they had under observation were definitely pathogenous to guinea-pigs, whereas Row, Parmanand, and Robertson reported no pathogenic signs whatever. Much the same divergence of opinion is found with regard to the

pathogenicity or non-pathogenicity of strains found naturally in mice or other animals. McDermott found that after a few passages the virulence of his strain became more or less stabilised with an average incubation period of nine days, and a duration of life of about twenty-eight days. Guinea-pigs showed the usual conjunctivitis and keratitis. Post mortem there was noted interstitial myocarditis, hyperplasia of lymphoid and endothelial elements in the lymph glands and endothelial proliferation in the spleen with progressive fibrosis.

Schockaert noted the occurrence of oedema in certain skin areas, e.g. of the eyelids, nostrils, lips, bases of the ears and genitalia. There were frequently areas of alopecia in the same situations and this was accounted for by a localisation of the *Spirilla*. The alopecia varied in extent and more especially in degree and was mostly transitory, lasting for fifteen days to five weeks. At autopsy Schockaert noticed haemorrhages and hyperaemia in the lungs, liver, kidneys and suprarenals. McDermott draws attention to the occurrence of latent spirillosis, first described by Mooser (1926), in which there are no symptoms and no appreciable signs of the disease except involvement of the lymph glands. Grabow and Struwe (1929) report certain differences from the findings of Mooser, but they were more a question of degree than a true distinction.

The changes occurring in the structure of the blood, bone marrow, lymph glands and spleen in guinea-pigs experimentally inoculated with rat-bite fever have been studied by Ishizu (1927). This worker found that though there was a distinct leucocytosis the lymphocytes showed a diminution both relative and absolute. There was, however, an increase in the monocytes and pseudo-eosinophiles. The red blood cells fall gradually in number as also does the haemoglobin percentage. Changes may occur in the red blood corpuscles themselves resulting in poikilocytosis, anisocytosis, polychromasia and punctate basophilia. In the spleen there is increase of the reticulo-endothelial system but the malpighian bodies are somewhat atrophied. The lymph glands of the mesentery showed atrophy of the capsule while in the centre of the gland there was a proliferation of the reticulo-endothelial cells and an increase of the plasma cells. The bone marrow as seen in the femur contained few erythroblasts but, on the other hand, there were numerous pseudo-eosinophile myelocytes.

Rabbits. Matsumoto and Adachi (1923) injected rabbits intra-scrotally with the formation of lesions similar to those of syphilis in that animal. After three to five days incubation the ' primary ' lesion appeared, followed by inflammation of the glands. Seven days after the appearance of the local condition signs of generalisation became evident. The course followed by the infection is very similar to that observed in guinea-pigs. Grabow and Struwe (1929) were able to demonstrate the patches of alopecia in albino rabbits but not in guinea-pigs. Immune bodies were found in the blood of rabbits and these observers are of the opinion that the immunity of recovered animals is dependent on these antibodies and not on the persistence of the infection.

Other Animals. Inoculations into fowls were performed by Schockaert with negative results except that the serum of one bird six weeks after inoculation contained powerful lytic substances. In monkeys, rat-bite fever seems to follow a course very similar to that in man (Futaki *et al.*; Ishiwara *et al.*; Kitayama and Mukoyama, 1917 ; Kobayashi and Kodama ; Ruys). Experimental inoculations have been performed on cats by Mooser (1925), Kasai (1923), and McDermott (1928), the last of whom found that although *Spirilla* were not demonstrable in the blood by direct microscopical technique their presence could be proven by inoculations into mice. Two ferrets also were infected by this worker.

Man. Inoculations with the virus of rat-bite fever have been performed on man as a therapeutic measure in cases of general paralysis of the insane. This has been done in America by Theiler and colleagues (1926) and in Europe by Schockaert (1928). It is not within the compass of the present work to discuss in detail the effects of the treatment. Suffice it that the results do not appear to be in any way superior to those obtained by induced malaria. More recently Hershfield, Kibler, Colby, Koenig, Schmid, and Saunders (1929) have described the effects of treatment with injections of *Spirillum minus* in seventy-two cases of paresis. Ten patients died during the treatment, but only two of the deaths were attributable to the rat-bite fever. The height of fever was not proportional to the degree of improvement evidenced by the patients for some in whom the temperature never rose unduly showed more improvement from the point of view of mentality than others with very high temperatures. A year after treatment 50 per cent. of the

patients showed varying degrees of improvement in the physical condition, while 20 per cent. showed slight to marked mental improvement. About 10 per cent., although at first showing improvement in the mental condition, later relapsed.

These inoculations into man have shown conclusively (Schockaert) that man is susceptible to the *Spirillum* no matter whether the organism is derived originally from a sodoku case or from a natural strain in animals. Experimental sodoku does not differ from that following the bite of an animal. The ' primary ' lesion may be prevented if the injection is intravenous and care is exercised to avoid contamination of the skin.

In man, apart from the ' primary ' lesion with its accompanying lymphangitis and lymphadenitis, no highly characteristic pathological lesions have been described. Rather are the findings, in such cases as go on to a fatal termination, similar to those which might occur in almost any febrile condition. One of the earliest accounts of a post mortem examination was that of Miura and Toriyama (1897). The case was one of a woman aged 32 who died on the 70th day after the bite. The cerebro-spinal fluid was increased in amount and the meninges showed a degree of hyperaemia. In the lungs a slight amount of inflammatory oedema was noted, but apart from this no other visceral lesion was detected.

The case recorded by Blake (1917), which has already been referred to above, was of interest in that a *Streptothrix* was isolated from the ulcerative endocarditis on the heart valves. In addition there was myocarditis with degeneration and necrosis of the cardiac muscle fibres. In their two cases Kaneko and Okuda found the same cachectic condition as was noted above. The viscera were hyperaemic and the liver and kidneys showed parenchymatous degeneration. In the bladder there was some slight cystitis and the stomach and intestinal mucosa showed signs of catarrhal gastro-enteritis. The heart muscle had evidence of myocarditis. It is obvious that the pathological records on human cases, especially as those which are available are from individuals showing great disparity in age and physique, are too scanty to permit of generalisation.

SEROLOGY

The Wassermann reaction has been tried by numerous observers on cases of rat-bite fever with a considerable proportion of positive results. ' If syphilis can be excluded,' says McDermott, ' a positive Wassermann reaction would be significant.' Surveyor and Row recorded negative results, while in the series of cases reported by Knowles and Das Gupta nine were tested, six were negative, one was doubtful, and two were ' moderately ' positive. These last two authors are inclined to doubt the diagnostic value of the complement deviation test. The work of Nakamura (1928) has been noted above.

Ito, Ido, Wani and Okuda (1917) were the first to demonstrate the presence in the serum of convalescents substances exerting a lytic effect on the organisms. In 1919 Kusama, Kobayashi and Kasai studied three strains of *Spirilla*, one from man, a second from a rat, and the third from a field mouse. The strains from man and the rat were pathogenic to guinea-pigs and monkeys but no symptoms were observed in these animals as a result of inoculation with the field mouse strain. Antibodies were not produced by the guinea-pig, the rat or the mouse. Serum from monkeys inoculated with the human and rat strains caused lysis *in vitro* of the three strains whereas monkeys injected with the mouse strain produced immune bodies only to the infecting strain. It was noted, however, that an infection with one strain immunised the animal against re-inoculation with either of the other two strains.

The divergence exhibited serologically by different strains is exemplified by the work of Kobayashi and Kodama (1919). Two strains were isolated from rats ; one of these was infective to guinea-pigs and monkeys and the serum of such animals after recovery was lytic for both strains whereas the other neither could produce a demonstrable infection nor did the serum show any lysis *in vitro*. Infection with the latter (milder) strain must have occurred for, subsequently, experimental animals were protected against inoculation with the more virulent organisms.

Kasai studied three strains, one human and the other two from wild and white mice respectively, in kittens. He found that the human and wild strains caused the presence in the serum of substances

lytic to all three, but from the white mouse strain a lytic effect was produced only in the same strain. Robertson (1924) demonstrated the presence of immune bodies in the serum of guinea-pigs which, though showing signs of disease, never had *Spirilla* in their blood. The immune bodies did not appear to be very powerful since in dilutions of less than one in four their effect was negligible. No signs of agglutination were observed although Yamada stated that this phenomenon did occur.

According to Schockaert the immune bodies are almost exclusively lysins, which, when the serum is only slightly diluted and is allowed to act on a heavy infection of *Spirilla* in the blood (four or five per microscopic field), are generally very active, so much so that the process actually may take place during the admixture of the infected blood and serum on the slide. In dilutions of 1 in 10 and 1 in 20 in physiological saline, which by itself has no influence on the organisms, the action of the serum is very feeble indeed and in 1 in 100 is unappreciable. Schockaert controlled his experiments *in vitro* by inoculating animals with infected blood which had been subjected to the action of lytic serum for one hour at room temperature (five drops of serum, three drops of blood). It was concluded that the serum in question was really active if, after the expiration of the normal period of incubation, infection had not taken place or if the onset was markedly delayed beyond the usual.

Schockaert carried out a most interesting series of observations on his cases of induced infection in man. He found that with a human strain derived originally from a rat-bite fever subject (Paris strain) the serum was practically equally lytic towards the same strain and also towards the mouse variety (Louvain strain). The appearance of the lysins in the serum would seem to take place between the fourteenth day after the first attack and one month. One case had only a faintly lytic serum on the fourteenth day—the infecting organisms were still present in the blood—while in another fifteen days immobilization and lysis (of both strains) was complete in three minutes. In contradistinction the mouse variety was inert (one case) or provoked the formation of lysins very faintly active to the human strain (the second case), but was extremely potent when in contact with the same strain. Infection in man with the Paris or the Louvain strain equally protected against re-inoculation with either

variety. According to Schockaert rats infected with the human strain never produced antibodies against either the human or mouse strains but those inoculated with the mouse strain formed lysins for the specific organism only. In mice the production of antibodies is extremely doubtful. Guinea-pigs certainly do produce antibodies. McDermott (1928) found that the serum of guinea-pigs at a late stage of the disease, in dilutions up to 1 in 8 was capable of immobilizing *Spirilla* in mouse blood in one hour no matter whether the strain was human or from mice. In rabbits antibodies were present after about ten weeks ; immune serum from such animals first immobilized and later caused lysis of the organisms in dilutions up to 1 in 16. The immune serum was also active against the mouse strain. According to Schockaert rats infected with the human strain never produced antibodies against either the human or mouse strains but those inoculated with the mouse strain formed lysins active against the specific organisms (i.e. these from the mouse strain) only.

The curative and protective action of immune serum has not been fully investigated although certain evidence adduced by Schockaert is highly suggestive. A dose of serum from a human case (1·8 c.c.) injected into a guinea-pig caused immediate disappearance of the previously numerous *Spirilla*. Death occurred—probably from the liberation of endotoxins—in a few hours. Inoculated mice, too, lost their infections (with either human or mouse strains) when treated with a dose of 0·5 c.c. of immune human serum. Mention has already been made of the findings of Grabow and Struwe (1929) who found immune bodies in the blood of rabbits and that the immunity of recovered animals is supposed to depend on these antibodies and not on a persistent (latent ?) infection.

Kuipers (1929) inclines to the belief that the cyclic course of rat-bite fever in man is to be ascribed either to a periodic rise in the production of toxins or to a periodical alteration in the relation between the toxins and the bodily resistance of the patient. This is based on the assumption that no cyclic alterations are known in the life of *S. minus*. (As has been noted above, Robertson (1924) drew attention to the fact that the numbers of the organisms in the peripheral blood of sub-inoculated animals was subject to periodical variations or fluctuations.) The rise in the number of eosinophile

leucocytes, which occurred in a case described by Kuipers and Ruys (1929), during the few days preceding an attack, is considered to be in the nature of a reaction to some substances produced by the infection.

CHEMOTHERAPY

Since the discovery in 1912 of the curative effect of salvarsan on human cases (Hata ; Surveyor ; Dalal), numerous drugs have been tried on man and animals, but none has yet been discovered which is to be preferred to the arsenical derivatives. It is possible that the antimonial stibosan may prove on further investigation to have a definite action. Bismuth and mercury have little effect on the course of the infection. Small doses of arsenical compounds do not cause the formation of arsenic resistant (drug fast) strains.

Schockaert (1928) has tested a number of preparations from the point of view of their action on experimentally induced *S. minus* infections in mice and guinea-pigs. The mice were treated during the second week of the infection when there were the greatest number of *Spirilla* in the blood. Tryparsamide, trypaflavine and germanin (Bayer 205) were found to have no effect. Arsenic in inorganic form had no effect on infected mice in dosages about 0·3 mgr. per 20 grams body weight. Organic arsenical preparations were more efficient. Atoxyl, for example, in a dosage of 1 mgr. per 20 grams B.W. and stovarsol (5·5 mgr. per 20 grams B.W.) caused a diminution in the number of organisms in the blood of mice but in guinea-pigs (in equivalent doses) they had no action. Sulfarsenol in dosages of 2·85 mgr. per 20 grams B.W. was sufficiently active to cause the disappearance of the *Spirilla* from a heavily infected mouse's blood for 13 days. Neosalvarsan was most remarkably active for, in doses of 2 mgr. per 20 grams B.W. the organisms disappeared for 15 days from the blood of mice while in guinea-pigs the same effect was obtained for from 4 to 10 days with dosages of 15 mgr. per kgr. B.W. One mouse was completely cured.

Four cases of induced sodoku in man (general paralysis of the insane) were cured with sulfarsenol, the highest dose of which was 36 cgr. Bismuth sodium tartrate had some action in mice, causing the organisms to disappear for one day. Tartar emetic had no

effect but stibosan (m. chloro-p acetylaminophenyl-antimoniate of sodium) in doses of 1·30 mgr. per 20 grams body weight caused the disappearance for 10 days in mice and for an equal length of time in guinea-pigs in doses of 55·5 mgr. per kgr. B.W.

Schwarzmann (1929) treated infected mice with salvarsan, but although the *Spirilla* disappeared from the circulation for a time, relapses occurred in at least 75 per cent. Abe and Shimoda (1927) used successfully oxyacetyl-aminophenyl-arsinic acid but Fischl (1929) found that solganal, solganal B, and lopion, all gold preparations, had no effect.

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PLATE XIII

EXPLANATION OF PLATE XIII

Spirillum minus Carter, 1887.

Photomicrographs at a magnification of × 2,500 approx. The specimens were stained with Tribondeau's modification of Fontana's silver nitrate impregnation method. The films were prepared from the peritoneal fluid of mice, showing a heavy infection in the peripheral blood. In the majority of instances the bodies appear to be thicker than they would in the unstained condition. In the centre are two dividing forms each bent on itself like the letter U.

BEHAVIOUR OF INFECTIVE HOOK-WORM LARVAE

BY

CLAYTON LANE

To infective hookworm larvae have been assigned a number of tropisms. Fülleborn (1924) quotes Przibram as defining a tropism, or rather a taxis, as the movement of an animal in a definite direction under the influence of a directive stimulus. But there can be no settlement of the fact or the extent of the tropisms of hookworm larvae without a clear picture of their behaviour in the absence of any directive stimulus.

Florence Payne (1923b) has greatly clarified our knowledge of their ordinary behaviour which, since they are obligatorily aquatic, dying if dried, can be observed only in water. She has pointed out, a fact readily verified, that they have alternate periods of activity and rest, the latter being a refractory state in which they will not react to stimuli, and which lasts from one to thirty minutes ; and that when at rest they are apt to assume ugly angular postures. The passage from rest to activity is usually gradual. This transition may chance to coincide with the application of a stimulus and lead to wrong conclusions, but if the change repeatedly occurs after a particular form of stimulation, it may fairly be attributed to that stimulus.

The writer has spent many hours* observing the behaviour of these larvae in different water conditions with or without the added influence of a possibly directive stimulus.

Before passing to findings and conclusions, it may be said regarding a possible hydrotaxis that an animal always enclosed in water as a condition of its life can hardly have a taxis to a body of water outside that in which it is living, however large the amount of the former may be. Moreover, if water in itself had any positive

taxis for a hookworm larva, it would not, when lying in. water, exhibit actions which suggest that it is doing its best to escape from that water, yet as will be seen that sort of movement is exactly what it does show. Nor can this be explained as a negative hydrotaxis, for no species which continues to survive can have an inherent, un-controlled impulse to get away from the element on which its life depends. Hydrotaxis, positive or negative, can be excluded from consideration in connection with hookworm larvae.

BEHAVIOUR IN A DEEP GLASS CELL

These cells are about forty times as deep as the length of the larva. They consist of cubical boxes of optically flat glass, each side measuring 25 mm., and of a cover of the same material. Water containing the larvae to be examined is placed in a cell and more water added till it seems full. The cover is then laid over about three-quarters of the cell, making contact with the water so that no bubble is enclosed. Should this happen, the box is slightly tilted and the bubble if possible made to glide into the uncovered part and to burst, helped, if necessary, by a hot wire. Should the bubble refuse to move, the cover is lifted off, wiped and replaced. When no bubble is imprisoned, water is, if necessary, added to the cell from a pipette till the uncovered surface appears flat, the cover then being slid into position. If in doing so an air bubble becomes enclosed, the cover must be slid back and more water added. For clear vision the box, when closed, must be completely free from any bubble. All manipulations should be carried out in rubber gloves and rubber apron. The optically flat bottom and sides of an accurately filled but uncovered cell allow of examination in one plane right up to its margins, which is not the case with any other vessel ; but .unless the surface of the water is quite flat the surface meniscus obscures clear vision of all parts near the edges, and even if complete flatness can be arranged in an uncovered cell, the rippling of the surface which occurs with every movement of the cell blurs vision everywhere. With the cover applied and no bubble enclosed there are sharp unwavering images of every part of the cell's floor under a low, say a two-inch, objective. They are indeed so sharp that the mirror

reduplication of larvae which lie close to the side of a box must not be allowed to give inaccurate larval counts when these are made.

If shortly after its filling the focus is adjusted well above the floor of the cell, larvae are seen either quiescent or in sinuous graceful movement, drifting about in the currents caused by the filling. That in either case they are merely drifting is clear from the parallel movements of the just-visible specks of other matter also suspended in the water.

While these indications of water currents are being watched, three things will be noticed. First, a straight, living, but motionless larva as it drifts along gradually takes up a position head-on to the current, as is shown by the way the drifting specks pass it ; for of course nothing immersed in water ever drifts quite so quickly as the current which bears it along, and the larger the body the greater the lag. The cause of the larva's altering orientation lies in its shape which places its centre of gravity in front of its equator, so that, just as does a weather vane, it must by the friction of the passing water passively head into the current. The same effect is seen as its own weight carries such a larva through the water to the bottom. It slowly turns head downwards, and time after time it will be found that the part which first touches the bottom is the anterior end. Second, a dead larva, recognised by its loss of sheen and its coarsely granular contents, remains suspended more readily than a live one ; some may be found drifting about in the upper part of the cell long after all living active or passive larvae have fallen to the bottom, as they all do after a relatively short time. Dead larvae are, then, lighter than living larvae and more susceptible to the action of currents. Third, a suspended active larva, moving with its usual rather slow grace, seems to head in no particular direction, nor do its movements result in appreciable progress through the water. Evidently these slight currents produce no directive stimulus, no rheotaxis, and the slight friction between the water and the smooth sides of the larva give the latter little purchase.

As soon, however, as a larva reaches the bottom, the added friction, smooth as the glass is, allows the larva to progress slowly head first, and this advance will bring it in time to the glass sides of the cell. But there is no more justification for supposing that the glass side has attracted it there, than for suggesting the same

of an obstructing wall against which a blind man hits his face. After all, the glass floor has been far nearer to it all the time.

On being brought to a standstill by the side of the box, larval movements often become more energetic and are very persistently directed roughly at right angles to the side wall of the cell. Though they may now be described as boring in character they are, as is fully recognised, essentially the same as those which brought the larva there, though perhaps of increased intensity; yet they must be accepted as now coming under Przibram's definition of a taxis, since they are directed roughly at right angles to a surface with which the larva is in contact and which, by its opposition to advance, provides a stimulus to which it continues to react. The movements at this stage fall in with the definition given by Fülleborn of a thigmotaxis, namely a directive stimulus produced by contact with a stable body. The stimulus is indeed so definitely directive and so successful in retaining the larvae at the side of the cell that the quickest way to determine their presence in suspected fluid is to examine first the edges of the cell floor after larvae have been given time to reach them.

These sinuous movements affect, however, the whole larva including its head, so that this is not maintained exactly at right angles to the side of the box against which it butts; accordingly the larva sideslips. These sideslips are rarely equal in both directions so that nearly always a larva can be observed sideslipping mainly in one direction until it reaches a corner. To move away from a corner would now be to move backwards and this action I have never seen effected by a free-living hookworm larva although I have watched many hundreds. Within bodily tissues Looss (1911) has reported such tail-first retreat, but it must rarely occur outside the body. When many active larvae have been placed in a cell their sideslips eventually produce a striking mass of wriggling individuals filling the corners. As has been noted, the glass forming the distant sides of the cell cannot have a greater attraction for a larva than the glass of its floor with which the larva is in close contact from the time that it falls to the cell's bottom, nor can the angle formed by two sides overwhelmingly attract larvae into it. These, therefore, reach the sides of the cell simply because their instinct for progression has in time carried them on till they can get no further; they stay at the sides because the resistance these offer to their progress

induces a thigmotaxis ; and they reach the angles not because they like to work or play in company, not because they have any herd instinct, but because the sloping walls lead each of them there mechanically, the corners having as little attraction for them as has the keddah for the herd of elephants which has come within the converging arms of its approaches.

Mechanical Activation.

Larvae react to certain stimuli. Tapping the cell gently on the bench will usually activate them, as will their transference from one vessel to another. For fifty years it has been known that heat activates larvae, that its effect is temporary even when the temperature is maintained, and that if reapplied within a short time it is less effective than it was at first. If a cell is placed on a water stage whose temperature as shown by its thermometer is raised to 45° C. (the contents of the cell not, however, reaching that temperature) the larvae within it become violently active, and may even leave the cell's floor to some extent ; though to what degree the strong currents, and to what the great activity, induced by the heat are respectively responsible for this lift, it is not easy to determine. If the hot stage is laid on the top of the cell, the larvae at its bottom remain there unactivated.

The writer has never been able to repeat the clear-cut results reported by Khalil (1922) in which larvae swam upwards to, and remained at, a heated point in the upper part of a vessel of water ; nor to convince himself that there are convention currents of a kind which will lift precipitated fuchsin and yet leave unlifted the easily raised dead larvae. Fülleborn seems to be of the same opinion since he lays stress on the very disturbing currents which are set up on attempting to investigate thermotaxis by the local heating of water ; currents so confusing that he for his part studied larval movements on an agar dish heated at one point, by placing tufts of strongyloides larvae upon it 1 cm. to 2 cm. from the heated part. He reported that most of them moved at the ' fabulous ' rate of 1·5 cm. in 10 seconds towards the heated point, that if this became excessively heated they remained fixed in a half circle round it, but that some individuals swerved back from it at the right moment. This last he correlates

with Khalil's observation that in his experiments some larvae at the edge of the vessel remained unattracted by the heated point.

Since some larvae refused to show any thermotaxis and others turn away from the point of heat, is not the explanation of their general activity something else than a thermotaxis? Is not activation by heat a sufficient explanation of the phenomena observed in this solid medium, acting in some such manner as the following? When larvae are placed at one point in such a petri dish and heat applied to another they will mostly become active as the heat reaches them. So long as their activity happens to carry them towards the source of heat, the rising temperature they experience will make them increasingly active and they will move towards the source of heat with increasing rapidity, so that within a given time many will chance to reach it. As soon as their activity begins to carry them past or away from the source of heat their movements will rapidly slacken with the fall in temperature, while if it have not originally taken them towards the source of heat their progress from the beginning will be slow and slight. Accordingly at a certain interval after they have been placed on the agar dish the only part where those larvae, which have left the site of inoculation, will be found agglomerated will be at the point of application of heat. This consideration implies that, could there be produced mechanical larvae provided with quite erratic movements which increased in energy as the temperature rose to a certain point, and then ceased abruptly as it passed that point, there would, it seems, be produced just such a distribution and behaviour as that described by Fülleborn.   •

All these considerations seem to justify an expression of disbelief that any sure evidence of thermotaxis has yet been offered, since the purely mechanical results of water currents or of activation by heat seem to explain all the phenomena, general and exceptional, more satisfactorily than does a thermotaxis.

Chemical Activation.

Looss (1911) held that after their second ecdysis sheathed larvae received no further nourishment. It has been noted above that their passage from quiescence to activity is sudden and unforeseen, and that it may chance to coincide with the application of a supposed stimulus

and yet be independent of this. In the writer's experience activation has too frequently occurred after the addition of serum (not obtained free from red corpuscles) to the fluid in which sheathed larvae lie for this to be fortuitous. This implies that some substance in serum has penetrated the sheath. If this conclusion is correct, substances which nourish larvae may also penetrate the sheath ; and this may explain the very varying degree of longevity reported for infective larvae by different observers, and the fact that their life has sometimes been very greatly prolonged.

BEHAVIOUR IN A CAPILLARY TUBE

A capillary tube is so fastened to a slide with plasticine that an up-turned expansion at one end is capable of receiving larva-containing water from a pipette, and a down-turned portion at the other is capable of being temporarily or permanently narrowed to regulate the rate of flow through the apparatus, which itself is so arranged that the capillary portion is under the microscope. After filling the apparatus with water at about 35° C. to the exclusion of bubbles, active larvae are added to the expanded part. If the relation between the head of positive water pressure at one end, and of negative pressure and the size of the exit at the other, have been happily balanced, active larvae will be seen under the microscope steadily heading up stream. It will further be noticed that as soon as a larva ceases to be active it is forthwith carried off down stream and passes out of sight in a flash. The larvae then which remain in view under the microscope are merely those which happen to be moving in the direction and at the rate which will keep them under this particular form of observation. That they are seen moving up stream in conditions which forbid of their observation unless they are doing so is not satisfactory evidence of a rheotaxis. To establish the existence of this would seem to require much more complicated experiments. Further, as has been noted, observations on larval behaviour in a cell disclose no active reaction to currents of the magnitude there met with, but do show that an upstream position will tend to be called into being mechanically by the shape and balance of the larvae.

BEHAVIOUR IN A FILM

In a considerable drop of water on a slide larvae behave as they do in a cell; their progress on the slide is slow and relatively ineffective. But, if active, they eventually reach the margin of the drop, and, as in a cell, persistently butt against this edge, which is held together in this case by surface tension. There is no more reason to suppose that their collection at the edge is evidence of a margin taxis than to maintain that their exactly similar aggregation against the glass sides of a glass-floored cell is evidence of a glass taxis. Nor can it properly be attributed to an oxygen taxis since oxygen in the atmosphere overlying them is necessary neither for their life nor their activity. Thus Boycott (1905, 1911) found that infective larvae retained the most lively movements for three weeks under hydrogen or nitrogen, and for at least eight days under carbon dioxide or in a vacuum—that is, under water vapour. These positive observations must outweigh those of Plett in Fülleborn's laboratory, who found indeed that carbon dioxide produced a negative taxis and that hydrogen caused cessation of activity; but stated that if exposure to hydrogen and to air were several times alternated, larvae no longer ceased activity under hydrogen. In explanation of the facility with which larvae dispense with oxygen, Looss (1911) suggested that they obtain their supply of this by some chemical change associated with that absorption of their constituent granules which accompanies their activity.

All these considerations lead, then, to the conclusion that the arrival of larvae at the margin of a deep drop of water requires for explanation the action of no taxis of any kind, but occurs merely because in their blind diffusion they can get no further; having reached it, they are retained in its neighbourhood by thigmotropism.

Moreover, as Fülleborn has noted, an observation readily confirmed, larvae collect in any tongue-shaped extension or in any angle of the margin of the drop, and he particularly mentions an angle where the water is skirted by air on one side and vaseline on the other. But their presence there should be explained, not as caused by any special taxis or because they are companionable creatures, but as the mechanical result in identical conditions and on a series of individuals of active movement, of thigmotropism, and of sideslip. They

collect because the same mechanical conditions guide each larvae to the same spot. The phenomenon is exactly the same as is seen at the angles of a glass cell and is explicable in the same mechanical way.

These are the conditions seen in a considerable drop of water. As this lessens by evaporation, two phenomena may be observed. With every shrinkage of the drop towards its centre, the tendency is for the surface tension of its edge to drag the larvae mechanically inwards towards its centre so that they become concentrated in the lessening pool ; though if any part of the drop gets left behind by irregular shrinkage it may contain larvae. On the other hand, as the drop becomes shallower and its upper surface with its surface tension approaches the slide, a larva evidently obtains a purchase between the two, for, if already at the margin of the drop, it may no longer butt against this ineffectively but by its thigmotaxis may burst through, and may progress along the slide until the slight film of water which it has carried with it dries up and it perishes. If the larva is not near the margin of the preparation, the purchase obtained against the now approximated upper and lower surfaces permits of rapid advance. Thus, in these conditions, and at room temperature, the writer has observed a larva escape from its sheath in a thin film of water such as that now considered and travel with markedly leisurely movements for 12 mm., measured in a straight line from the discarded sheath, in ten minutes ; after which it passed into a phase of quiescence. This is at the rate of three inches an hour. As noted above, Fülleborn has observed with a lens on agar, and under the stimulus of heat, a tuft of strongyloides larvae moving at the rate of 1·5 cm. in ten seconds, or 5·2 m. (6 yards) an hour. He himself speaks of the rate as ' fabelhafter ' ; it is perhaps partly caused by a diffusion on and in the agar of the fluid in which the tuft was necessarily contained. But in favourable conditions active migration of larvae is evidently considerable and the writer's observation on the rate of active progress made by this leisurely moving larva clearly does not measure the capacity for migration of one which is in energetic action. Further, Fülleborn has shown that active advance of strongyloides larvae is not influenced by any barytaxis (or geotropism), positive or negative, but apparently occurs with equal facility in all directions, upwards, downwards or sideways ; and the same presumably holds for hookworm larvae.

As regards the distance which has actually been covered in nearly natural conditions, Florence Payne (1923a) buried hookworm larvae under three feet of sandy loam in a vertically set pipe 12 inches in diameter. They became visible on the surface to a hand lens in fifteen days. No greater depth was investigated and this experiment was closed after eighteen days, but there is no reason to suggest on the one hand that larvae cannot penetrate more than three feet of soil or on the other that they cease to wander after eighteen days. At the termination of the experiment one larva in 400 had reached the surface layer, while 1 in 50 was discovered by water extraction somewhere between this and the place of burial. Those not found were probably dead or had escaped through one or other end of the pipe, which was provided with no larval trap. It is clear that the capacity for diffusion of mature larvae is great, and that their radius of action is essentially determined by the physical conditions of their environment, of which the size of soil particles and the arrangement of water films round them are particularly important.

While this behaviour is needful for the survival of the species, it may lead individual larvae into situations in which their death is inevitable. This is of little import to the species since the progeny of a single pair of necators would in optimum conditions number over 15,000,000,000,000,000,000,000 at the end of one year, and of ancylostomes apparently many more ; but such behaviour has this importance in preventive medicine that there should be a possibility of devising traps which would use it for the self-destruction of hookworm larvae as they escape from certain types of imperfect latrines.

CONCLUSIONS

These observations and this reasoning lead then to the conclusion that there is no adequate evidence for the existence in hookworm larvae of any taxis other than thigmotaxis, the result of actual contact, and that observations which have been held to suggest the contrary are explicable on purely mechanical grounds. They lend little countenance to any departure from what seems to have been Looss's opinion. They may be summarised conveniently if dogmatically by the statement that the behaviour of a larva is not complicated.

It comprises automatic movement of a kind which will enable it to reach its normal host ; movement which is capable of intensification by disturbance through that host's foot, by the heat of his body and perhaps by his specific body fluids, and of conversion into a thigmotaxis by the resistance to entry offered by that host's skin. Nothing more seems necessary to explain observed larval behaviour, and nothing more to be implicit in it as observed.

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TRICHOSTRONGYLUS CAPRICOLA Ransom, 1907, FROM THE GOAT IN ENGLAND

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During investigations on the morphology of the genus *Trichostrongylus*, the writer came across two bottles containing a large number of worms. One of them was kept in the museum of the Liverpool School of Tropical Medicine and was labelled: ' Worms from upper ileum of Goat 135. 11.1.21.' and on a separate label ' *T. instabilis* '. The other bottle was kindly lent by Mr. A. W. Noel Pillers, F.R.C.V.S., and was labelled ' Worms from debris of lower ileum. Goat 135. 11.1.21.'

On referring back to the books in the School it was found that the goat 135 was received on 18.12.1920 from Mr. Boyden, of Bromsgrove, England, for Mr. A. W. N. Pillers, and it was stated that the goat was infected with round worms. The goat was later killed owing to emaciation and was found to harbour, together with other worms, Trichostrongyles in the duodenum, jejunum, and ileum.

On examining the worms the writer found that they were a mixed infection of *T. capricola* Ransom, 1907, *T. colubriformis* Giles, 1892, Ransom, 1911 (= *T. instabilis*), and *T. extenuatus* (Railliet, 1898) Ransom, 1907.

The writer made an exhaustive search in the literature for the record of *T. capricola* in Great Britain, with a negative result ; the species of this genus so far recorded in Great Britain for ruminants (mostly sheep) are only *T. colubriformis* (Giles, 1892), Ransom, 1911, *T. vitrinus* Looss, 1905 and *T. extenuatus* (Railliet, 1898) Ransom, 1907.

Furthermore, the diagnosis of the writer of *T. capricola* was confirmed by comparing this species with co-types kindly lent by Dr. M. Hall, of U.S.A. Bureau of Animal Industry, Department of Agriculture. The writer therefore takes this opportunity of recording *T. capricola* in goats for the first time in England, as it appears (like other members of the genus) to be of both pathological and economic importance.

A full account of the morphology, hosts infected and geographical distribution of the genus will follow in a later publication.

THE DOMESTIC MOSQUITOS OF GADAU, NORTHERN NIGERIA, AND THEIR RELATION TO MALARIA AND FILARIASIS

BY

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The following report concerns results obtained from the regular examination and dissection of domestic mosquitos from the Tsetse Investigation headquarters at Gadau, Northern Nigeria, during the period June, 1929-March, 1930. It is regretted that pressure of other work necessitated absence from the station during November and December, 1929, and made it impossible to deal with several important points with relation to mosquito breeding and aestivation during the dry season.

I. SITUATION

The Tsetse Investigation headquarters at Gadau is situated on a high sand ridge four miles south of the Jemaari River, and eleven miles north of Azare. The surrounding country consists of undulating sand ridges, replaced towards the river by a low-lying mud plain which is usually more or less flooded during the rainy season. On the high ground the vegetation is of an open type with low thorn scrub and scanty Baobab, and much has been replaced by farm land. The natural vegetation on the river plain is heavier Baobab bush with dense thickets leading to heavy fringing forest along the river banks.

The Jemaari river flows for about six months in the year, and during the dry season the only open water in the district is confined to pools of varying size in the river bed or near it. From December to June the nearest open water to Gadau is at a point on the river

three and a half miles from the station. During this period the inhabitants of the neighbouring villages rely entirely on deep wells for their water supply.

II. CLIMATE

The climate is dry, with an average annual rainfall of 28 inches, all of which falls between April and October. The meteorological records for 1929 are given in Table I.

TABLE I

Meteorological Records, Gadau, 1929

Month	Mean Temperature	Absolute Maximum	Absolute Minimum	Relative Humidity	Rainfall	Prevailing Wind
January	68·7	87	51	34	o	N-NE
February	77·4	93	54	30	o	N-NE
March	85·43	102	67	30	o	N-NE
April	88·8	102	72	33	o	N-NE-W
May	88·6	103	75	55	1·87	W
June	82·1	100	68	60	4·28	W
July	78·8	92	66	76	6·35	W
August...	77·2	90	66	77	11·74	W
September	79·5	94	65	78	2·90	W
October	77·1	99	54	68	0·91	W
November	73·4	99	49	50	o	W-NE
December	67·1	92	39	—	o	N-NE

During the rainy season the prevailing wind is West. In November the North-Easterly Harmattan wind commences and blows continuously until April. During this Harmattan period the humidity falls to a very low level and the daily variation in temperature is great.

III. THE DOMESTIC MOSQUITOS

Table II summarises the information obtained from identification of daily catches of female Anophelines from the European houses.

TABLE II

Anophelines taken in daily catches from European bungalows : Gadau, 1929-1930

Species	1929						1930				Percentage of all species
	June	July	Aug.	Sept.	Oct.	Nov.-Dec.	Jan.	Feb.	Mar.	Total	
A. costalis Theo. ...	2	86	567	275	73		2	—	2	1,007	47·2
A. funestus Giles ...	—	9	194	329	387		13	11	1	944	44·2
A. squamosus Theo.	—	—	49	22	8	Away from Station	—	—	—	79	3·7
A. pharoensis Theo.	—	—	19	25	1		—	—	2	47	2·2
A. mauritianus Grp.	—	—	10	11	15		—	—	—	36	1·7
A. rufipes Gough ...	—	1	5	3	4		—	—	—	13	0·6
A. nili Theo. ...	—	1	4	1	1		1	—	—	8	0·4

The domestic Culicines recorded consist of the following species : *Culex nebulosus* Theo. ; *Taeniorhynchus (Mansonoides) uniformis* Theo. ; *Lutzia tigripes* Grp. ; *Culex decens* Theo. ; *Aedes aegypti* Linn. ; *Stegomyia vittata* Big. ; *Aedes ochraceus* Theo. During the whole period only 74 Culicines were taken in the houses, as against 2,134 Anophelines, a percentage of 3·4. *C. nebulosus* made up nearly half of this total.

The percentages of domestic Anophelines agree with those obtained by W. B. Johnson in Northern Nigeria (1919). *A. squamosus*, *A. pharoensis*, and *A. mauritianus* proved rather more numerous than in the stations where this author made his collections. It is noteworthy that the proportion of *A. costalis* to *A. funestus* varied through the rains. The former species appeared early in the rains and increased in numbers until August, the month of heaviest rain ; thereafter its numbers diminished steadily. *A. funestus* appeared later and was most numerous in the early dry season before the onset of the Harmattan winds. After December mosquitos are rare, though a very limited amount of breeding appears to go on, as the few specimens obtained at this time had the appearance of being recently emerged flies.

Lack of time prevented much search being made for the larval stages of the domestic mosquitos, and such information as was obtained contained no new data as to breeding grounds.   Larvae of most of the Culicine mosquitos mentioned above and of *A. costalis* and *A. funestus* were taken during the rains on or near the camp and in marshes on low-lying ground within a mile of Gadau.   Larvae of the rarer domestic Anophelines were not seen, though no prolonged search was made for them, and it seems probable that much of the breeding of some of these species (e.g. *A. nili*, *A. pharoensis*, *A. mauritianus*) takes place on the flooded plain and backwaters of the Jemaari river.

IV. MOSQUITO DISSECTION

Between June, 1929, and March, 1930, a total of 3,563 female Anophelines and 117 female Culicines were dissected and examined for parasites.   All of this material was obtained from dwelling-houses, both European and African, in Gadau.

The technique employed was that described by Stephens (1911) for the examination for sporozoites and zygotes of *Plasmodium*, with the addition that the proboscis and the teased-up thoracic muscles of each mosquito were also mounted and examined for Filaria larvae.

With regard to the zygotic index, the figures given below are obtained from those specimens in which digestion of the blood meal had proceeded sufficiently to allow complete examination of the mid-gut wall.

The salivary infections with *Plasmodium* sporozoites were usually heavy, while the zygotic infections varied considerably.   In Filarial infections the thoracic and cephalic muscle infections were usually heavy.   The number of mature worms in the proboscis varied from one to four.

V. MOSQUITOS IN RELATION TO MALARIA

The malarial index amongst the African population in the Northern Provinces is interesting in that it shows very considerable seasonal variation.   H. Morrison (1927) at Kaduna examined large numbers of blood films and showed that the monthly malarial incidence rises as high as 33 per cent. in July, in the early rains ;

there is a fall during the heavy rains in August and September to
22 per cent., a second high point of 26 per cent. in October, and
thereafter a rapid fall to under 5 per cent. in January. The infection
rate remains under 5 per cent. until the break of the rains in April.
Apart from the obvious relation between malarial incidence and
Anopheline abundance which accounts for the low malarial incidence

Chart to show fluctations in the Sporozoite Rates in *A. costalis* and *A. funestus* at Gadau, June-October, 1929.

Continuous line ———— *A. costalis.*
Interrupted line - - - - - - *A. funestus.*

The above percentages are based on average weekly dissections of 113 *A. costalis* and 77 *A. funestus.*

during the dry season, there is also a close coincidence between the
fluctuations in the sporozoite index of Anophelines during the
rains (see Chart) and the malarial index during the same period.
This is evidenced by the high point in the early rains, the fall during
the heavy rains, the secondary rise in the late rains and the sub-
sequent fall.

Malaria in the Northern Provinces appears to be chiefly of the subtertian type. Morrison (*op. cit.*) found that 93·8 per cent. of 292 malarial infections in Africans at Kaduna were subtertian, 5·5 per cent. quartan, and 0·7 per cent. benign tertian. In the dissections recorded below no attempt was made to differentiate the infections in the mosquito.

The only two Anophelines in which developmental forms of Plasmodium were found were *A. costalis* and *A. funestus*. The other species of domestic Anophelines and the numbers dissected are as follows : *A. pharoensis*, 205 ; *A. squamosus*, 83 ; *A. mauritianus*, 46 ; *A. rufipes*, 24 ; *A. nili*, 9. Neither sporozoites nor zygotes were found in any of these species. Table III gives the monthly sporozoite and zygote infection rates in *A. costalis* and *A. funestus*, while the chart shows in graphical form the weekly fluctuations in the sporozoite rate in these two species.

TABLE III

Monthly Malaria Infection Rates in *A. costalis* and *A. funestus* at Gadau (in percentages).

Month	Species	Number examined	Sporozoite Rate	Zygote Rate
June, 1929	*A. costalis*	20	15·0	28·6
June, 1929	*A. funestus*	8	0	0
July, 1929	*A. costalis*	396	12·6	17·1
July, 1929	*A. funestus*	89	7·9	15·3
August, 1929	*A. costalis*	688	5·2	12·1
August, 1929	*A. funestus*	259	3·5	8·8
September, 1929	*A. costalis*	659	6·5	9·6
September, 1929	*A. funestus*	421	3·8	7·3
October, 1929	*A. costalis*	169	6·5	9·1
October, 1929	*A. funestus*	457	2·6	2·3
November, 1929	*A. costalis*	—	—	—
December, 1929	*A. funestus*	—	—	—
January, 1930	*A. costalis*	4	0	0
March, 1930	*A. funestus*	26	0	0

Mosquitos in appreciable numbers first appeared in dwelling-houses during the second week in June, three weeks after the first heavy rain of the year. The malarial infection rate (both sporozoite and zygote), in these first mosquitos was high and the sporozoite infection rate reached a maximum of 18 per cent. for *A. costalis* and 12·5 per cent. for *A. funestus* in the second half of July. During the heavy rains of August the infection rate in both species fell sharply and reached a minimum of 2·4 per cent. for *A. costalis* and nil for *A. funestus* at the end of this month. With the slackening off of the rains in September the infection again mounted to 9·2 per cent. in *A. costalis* and 5·4 per cent. in *A. funestus*. Following late rain in the first week of October there was another fall followed by a small rise. From January to March, 1930, no infections either of sporozoites or zygotes were found in the specimens examined. The fluctuations in the infection rate during the rains are similar in both species and evidently are caused by the proportion in the catches of newly-emerged flies which have not had time to acquire infections. This proportion depends directly on the rate of breeding which itself depends on the rainfall, and reference to the chart (p. 429) shows this relation between rainfall and infection rate very clearly.

It was one of the objects of this work to contrast the malarial infection rate in Anophelines from the European and African quarters. In Gadau the African camp is situated at a distance of 500 yards from the European station. Daily catches of Anophelines were made from houses in both African and European quarters, and the results of their dissection and examination are summarised in Table IV

TABLE IV

Summary of Sporozoite Infection Rates in *A. costalis* and *A. funestus* : Gadau, June 1929-March, 1930 (in percentages).

Origin				Species		Total number examined	Sporozoite Rate
African Compound	...	...	...	*A. costalis* ...	...	1,174	7·49
,, ,,	...	...	...	*A. funestus* ...	...	554	4·15
European Station	...	...	...	*A. costalis* ...	...	762	7·22
,, ,,	...	...	...	*A. funestus* ...	...	706	2·97
All sources	...	...	...	*A. costalis* ...	...	1,936	7·39
,, ,,	...	...	...	*A. funestus* ...	...	1,260	3·49

The sporozoite infection rates in *A. costalis* from the two sources are nearly equal, while in *A. funestus* the specimens from the African camp proved to be 1 per cent. more heavily infected than those from the European quarters. The difference in infection is smaller than might have been anticipated, as quinine prophylaxis is regularly employed by the European staff. Furthermore, the servants' quarters are 100 yards from each bungalow and native children are absent, whereas in the African camp they are very numerous.

A comparison of the sporozoite infection rates in *A. costalis* and *A. funestus* over the whole period of the dissections shows that the former species is just over twice as heavily infected as the latter. The two species together must account for practically all malaria transmission in the Northern Provinces. The small extent to which other Anopheline species come into contact with man prevents their becoming of importance as malarial vectors. Apart from *A. pharoensis*, only very small numbers of the rarer Anophelines were dissected, and no opinion can be ventured on as to their potentialities as carriers of malaria in Northern Nigeria, no transmission experiments having been carried out.

VI. MOSQUITOS IN RELATION TO FILARIASIS

Filariasis is extremely common amongst the natives of Northern Nigeria. *F. bancrofti* and *A. perstans* are the species found most frequently, the former being most commonly seen in blood films. Dyce-Sharp (1928) has shown in the Cameroons that complete development of *A. perstans* takes place in *Culicoides austeni* and attempts by several workers to infect various species of mosquitos with this nematode have all failed. In the absence of evidence that other species of *Filaria* occur here, and failing any reliable and rapid method of identification of larval forms of *Filaria* in the mosquito, it is assumed that all filarial infections encountered in dissections of domestic mosquitos at Gadau are due to *Filaria bancrofti*.

Species of Culicidae in which mature infections with *Filaria bancrofti* have been found are *A. costalis*, *A. funestus*, and *Aedes*

ochraceus. Immature infections, in which only thoracic muscles are involved, were also seen in *A. pharoensis* (5 per cent.), and *A. squamosus* (1·2 per cent.). Table V summarises the infection rate for the whole period of dissections in *A. costalis* and *A. funestus.*

TABLE V

Summary of Filarial Infection Rates in *A. costalis* and *A. funestus* : Gadau, June, 1929-March, 1930 (in percentages).

Origin	Species	Number examined	Mature Filaria Rate	Immature Filaria Rate	Total Infection Rate
African compound ...	*A. costalis* ...	1,174	1·53	7·41	8·94
„ „ ...	*A. funestus* ...	554	0·54	3·43	3·97
European station ...	*A. costalis* ...	762	1·84	6·30	8·14
„ „ ...	*A. funestus* ...	706	1·13	2·97	4·11
All sources	*A. costalis* ...	1,936	1·65	6·97	8·62
„ „	*A. funestus*	1,260	0·87	3·17	4·05

It is found that *A. costalis* is a little more than twice as heavily infected as *A. funestus.* The total infection rate in *A. costalis* proved to be 8·6 per cent., with a proboscis (mature) infection rate of 1·65 per cent. In *A. funestus* the total infection rate was 4 per cent. with a proboscis infection rate of 0·9 per cent. Mosquitos taken in European and African quarters were infected to approximately the same extent.

A single specimen of *Aedes ochraceus* out of a total of 15 dissected was found to contain a mature infection. As this Culicine only forms 0·4 per cent. of the total number of domestic mosquitos examined it cannot be considered an important vector of *Filaria* in this district.

A. costalis and *A. funestus* in this part of West Africa are evidently efficient carriers of *F. bancrofti*, and in view of their overwhelming preponderance, both over other Anophelines, and over any domestic Culicine, they may be regarded as the principal vectors of this species of filaria in the Northern Provinces of Nigeria.

VII. OTHER PARASITES OCCURRING IN DOMESTIC MOSQUITOS

In the course of these dissections several species of parasites were encountered and they are considered separately below. No identifications have yet been made, and only a very general description is given in each case.

(a) Nematodes in body cavity and gut.

These were seen six times in the body cavity of *A. costalis* and once in the gut ; once in the body cavity of *A. funestus*, and twice each in *A. mauritianus* and *A. squamosus*. The number of nematodes in each case varied from one to three.

(b) Nematode ova (?) in ovaries.

These bodies are evidently identical with those described by Schwetz (1929) in *A. costalis* at Stanleyville, Belgian Congo. The infections were always massive and were confined to the ovaries, to which they gave a yellowish appearance. The individual eggs are ovoid with thick sculptured walls, and agree with the description given by Schwetz (*op. cit.*).

They were seen six times in *A. costalis* and twice in *A. funestus*.

(c) Spore-containing cysts on mid-gut wall.

These structures, possibly identical with those described by Schwetz (*op. cit.*), were frequently seen in *A. costalis* and *A. funestus*. Usually the cysts had been accidentally ruptured and the spores were scattered, but in a few cases the intact cysts were seen.

(d) Spirochaete infection of mid-gut.

One heavy infection was seen in a specimen of *C. nebulosus*.

(e) Herpetomonas infection of mid-gut.

One heavy infection was seen in a specimen of *L. tigripes*.

(f) Plasmodium praecox (?) zygotes on mid-gut wall.

One very heavy infection with small oöcysts was seen in an *L. tigripes* specimen taken in a European bungalow. The salivary glands were not infected.

VIII. SUMMARY

1. A short account of the situation and climate of Gadau is given. The climate is dry with a small rainfall which is distributed over about five months of the year.

2.   An analysis of the species of domestic mosquitos shows that *A. costalis* and *A. funestus* preponderate over all other species and together form 91·4 per cent. of the domestic Anophelines.   Culicines are very scanty and only form 3·4 per cent. of all domestic mosquitos.

3.   Mosquitos generally are only numerous from July to November.

4.   Dissections of 3,563 Anophelines and 117 Culicines taken in European and African dwelling-houses have been carried out, and analyses of the malarial and filarial infection rates are given.

5.   The infection rates with both *Plasmodium* and *Filaria* are approximately equal in the collections from the European and the African sections of the station.

6.   *A. costalis* and *A. funestus* are the only species of Anophelines proved to be concerned in the transmission of malaria.

7.   Considerable fluctuations in the sporozoite rate in *A. costalis* and *A. funestus* during the rains are shown to occur.   These are found to coincide with, and doubtless account for, similar fluctuations in the malarial incidence in the African population.

8.   *Filaria bancrofti* is shown to be transmitted primarily by *A. costalis* and *A. funestus*, both of which species are shown to be heavily infected, in both the European and the African sections of the station.

9.   A mature filarial infection was seen in *Aedes ochraceus*, and immature infections in *Anopheles pharoensis* and *A. squamosus*.

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THE INFLUENCE OF PASSAGE THROUGH THE INVERTEBRATE HOST ON THE BIOLOGICAL CHARACTERS OF PARASITIC PROTOZOA

BY

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In the course of chronic infections caused by protozoa and spirochaetes, these parasites show changes in their biological qualities, e.g. they lose their reactivity to serum antibodies and develop into so-called serum-fast or relapse-strains ; or, under the influence of chemical agents, into ' drug-fast ' strains. An animal that has passed the acute phase of the infection can be superinfected by a relapse strain, but no longer by the original strain.

What happens if a relapse strain or a drug-fast strain passes through the insect which as a rule transmits the disease in nature ? Does it keep the acquired quality or does it lose it, regaining the qualities of the primary strain ? The question is of considerable practical importance ; if the relapse strain passes through the insect unchanged, we must expect in nature an innumerable variety of strains of a parasitic species, differing from each other serologically and immunologically.

GONDER, REICHENOW and DUKE have tried to solve the question by using arsenic-fast strains, but their results are not in agreement. During an expedition to German East Africa, 1912-14, I and my late friend and collaborator HANS SCHRECK had the opportunity of experimenting on tsetse-flies (*Glossina morsitans*) and different strains of *Trypanosoma brucei*. The flies we caught on the mainland

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and brought over to Makatumbe, a small coral-island near the mouth of the bay of Dar-es-Salaam, where neither tsetse-flies nor indigenous vertebrates existed. There they deposited their pupae, and the flies hatched from them were used for the experiments, being certainly free from infection. Most of the experiments were carried out by SCHRECK. The trypanosomes we used were of different origins : one was an old laboratory strain from the Hamburg Institute of Tropical Diseases—strain ' *Hamburg alt* ' ; the second was a strain which I had secured from a Gnu killed in the bush and transmitted to guinea-pigs and white rats by *Glossina morsitans* in series : fly-animal-fly ; *strain ' Gnu.'*

(1) Strain ' Hamburg alt ' killed a white rat in about four to five days ; the trypanosomes increased in number continually from the first day after inoculation. When we sterilised mice infected with this strain by an arsenic compound, e.g., arsenophenylglycin, the mice showed an immunity against reinfection, lasting weeks or months. The strain thus showed antigenic properties.

Animals (goats, horses, or cows) infected chronically with Hamburg alt showed specific antibodies in their sera : if a mixture of serum and trypanosomes (Hamburg alt) in proper proportion was injected into mice, the infection did not take ; with other laboratory strains of *Tryp. brucei* the effect of the serum was zero.

(2) Strain Gnu was much less virulent than strain Hamburg alt A white rat, infected with blood from fly-dog-rat, lived 44 days, the next passage—rats 30 and 35 days. In vitro the serum immobilised the homologous trypanosomes within ten minutes to four hours and dissolved them. The antigenic properties of the strain, shown by the immunising effect after sterilising treatment(see above), was zero or nearly so ; an animal infected with the twelfth passage fly-rat-fly, with a feeble blood infection (+ +) cured by arsenophenylglycin and 5 days later reinfected, showed trypanosomes 6 days later and died 33 days later.

The antigenic property of the strain came out more clearly when the trypanocidal property of the serum of an infected animal was tested ; the serum of a bull infected by flies killed *in vitro* the homologous trypanosomes of strain Gnu within ten minutes, whereas an old laboratory strain (' Mesnil ' from the Pasteur Institute in Paris) was still alive after one hour ; and when the mixture of

serum and homologous trypanosomes was injected into mice, infection failed.

(3) We let the strain ' Hamburg alt ' pass through our laboratory-bred *Glossina morsitans*. The flies fed on a goat infected with Hamburg alt ; after more than three weeks they transmitted the trypanosomes to a dog. We called the new strain ' *Hamburg recens*.'

When this fresh strain was inoculated into white rats, the first trypanosomes appeared on the fifth day, increased slowly, reached their maximum about four days before death, and killed rats within 16 to 42 days. The virulence had therefore diminished considerably and was about equal to the virulence of the ' wild ' or ' bush ' strain Gnu.

Experiments showing the antigenic effect of killed trypanosomes (see above) were not made with the Hamburg recens strain ; but the serum experiments were conclusive.

Mouse No.	Inoculated with	Trypanosomes in the blood on						
		1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
1	Serum of goat 5042 (inoculated with ' Hamburg alt ') mixed with Hamburg alt-Trypanosomes	o	o	o	o	o	o	o*
2	,, ,, ...	o	o	o	o	o	o	o*
3	Hamburg alt-Tryp. only...	+	++	++++	Died	...	...	...
4	The same serum, mixed with Hamburg recens Trypanosomes	−	o	+	+++	+++	+++	+++*
5	,, ,, ...	−	o	+	++	++	++	+++*
6	Hamburg recens Tryp. only	o	+	++	++++	++++	++++	++++'

* No further notes reached Berlin after 1 June, 1914.

Every serum was heated to 45° Cels. for half-an-hour to kill the trypanosomes accidentally contained in it.

A young calf was infected by the bites of *Glossina morsitans* infected with Hamburg recens.

Mouse No.	Inoculated with	Trypanosomes in the blood on							
		1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day
1	Serum of calf (5556) mixed with Trypanosomes Hamburg alt ...	+	++	++	++++	Died	...	...	...
2	„ „ ...	+	++	++	Died	...	...	...	...
3	Hamburg alt only	+	+	++++	Died	...	...	...	...
4	Serum of the same calf, mixed with Tryp. Hamburg recens ...	−	o	o	o	o	o	o	•
5	„ „ ...	−	o	o	o	o	o	o	•
6	Hamburg recens only	o	+	+	++	++++	++++	++++	•

The strain Hamburg recens was inoculated into rats and brought over to Berlin. There other experiments were carried out.

Mouse No.	Inoculated with	Trypanosomes in the blood on					
		2nd day	3rd day	6th day	8th day	17th day	20th day
1	Serum of goat 6001 (infected with Hamburg alt) mixed with Hamburg alt	o	o	o	o	o	o remains free ...
2	Hamburg alt only	+	++	Died	...	...	...
3	The same serum, mixed with Hamburg recens	o	+	++	++	Died	...
4	Hamburg recens only	o	+	++	+++	++++	Died

Calf 6036 was inoculated in Berlin with strain Hamburg recens.

Mouse No.	Inoculated with	Trypanosomes in blood after					
		2 days	3 days	5 days	10 days	23 days	30 days
1	Serum of calf 6036 mixed with Hamburg alt	++	Died	...	...	...	...
2	Hamburg alt only	+++	Died	...	...	...	...
3	Same serum mixed with Hamburg recens ...	o	o	o	o	o	o
4	„ „ ...	o	o	o	o	o	o
5	Hamburg recens only	+	+++	Died	...	...	...

In vitro the serum killed the homologous trypanosomes within half-an-hour to four hours, whilst the heterologous trypanosomes were not more influenced than the controls.

The trypanosomes of strain Gnu behaved in different serum-experiments *in vitro* and *in vivo* exactly like the Hamburg-recens-Trypanosomes. This strain has recovered all qualities of a ' genuine ' or ' wild ' strain.

We have, therefore, given experimental proofs that

(1) The passage through the natural transmitting agent of *Trypanosoma brucei, Glossina morsitans,* has a decisive influence on the biological qualities of an old laboratory strain (virulence, reactivity to specific antibodies of serum). As this reactivity to antibodies is a characteristic of relapse strains, we may conclude, that *the passage through the tsetse-fly has changed a relapse strain into an original strain.*

(2) *That this changed strain is identical with a ' genuine ' strain recovered from a wild antelope in the bush, and kept in passages : fly-animal-fly.*

It seems possible that in this process of restoration the sexual phase through which the parasite passes in the fly may play an important part.

Our experiments are decisive on the question of immunisation against protozoan and spirochaetal diseases : if the insect host were to transmit any relapse strain unchanged, it would be necessary

to immunise against every possible relapse strain, which is practically impossible. But if the Brucei-strain of a certain district is a unit, we may hope—and our experiments in Makatumbe, 1914, gave us strong support—to immunise against this uniform strain.

The fastness of a relapse strain against the serum-antibodies is an acquired quality. It is lost by the passage of the parasite through the insect. So we have an example of mutative loss of an acquired quality : an interesting fact in heredity

'FOUADIN' AND 'AUREMETINE' IN THE TREATMENT OF *S. HAEMATOBIUM* INFECTIONS AMONGST WEST AFRICAN CHILDREN; TOGETHER WITH OBSERVATIONS ON THE AFTER-RESULTS OF TREATMENT WITH EMETINE PERIODIDE AND EMETINE HYDROCHLORIDE

BY

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AND

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I. TREATMENT WITH 'FOUADIN' AND 'AUREMETINE'

Our first experiments were carried out with ' Fouadin,' a preparation of Trivalent Antimony introduced by Messrs. Bayer & Co. They recommend that the drug should be given intramuscularly and that for a full course of treatment the dose in cubic centimetres should be about equal to the weight of the child in kilogrammes. This amount is to be given in ten doses, of which the third to the tenth are equal in amount and given on alternate days. The first three injections can be given on consecutive days and should be about thirty per cent. and seventy per cent., respectively, of the succeeding full doses.

Six West African children, aged seven to fifteen, were selected for treatment with ' Fouadin '. These children were attending a missionary school situated in Konno, Sierra Leone, a district heavily infected with *S. haematobium*. All six cases represented a severe type of the disease.

Below, in Table I, we summarise the results obtained with the prescribed course of 'Fouadin' treatment. In this, and all subsequent Tables, each finding represents the result of the examination of three cover-slip preparations made from the centrifuged deposit of ten cubic centimetres of urine.

TABLE I

Showing the results of 'Fouadin' treatment on six West African children severely infected with *S. haematobium*.

No.	Age and Sex	Weight in Pounds Before Treatment	Weight in Pounds After Treatment		Before treatment commenced	1	3	4	5	6	7	8	10	11	13	14	16	18	20	22	24	26	Total dose in C.C.
												Last day of treatment shown by double line (after 18).											
1	10 Male	72	68·5	Live ova	+	+	+	+	+	+	+	+	o	o	o	o	o	o	−	o	o	o	29
				Dead ova	o	o	o	o	o	+	o	o	1	2	o	o	o	1	−	1	o	o	
2	10 Male	61	60·5	Live ova	+	+	+	+	+	+	+	+	+	−	+	+	+	+	o	o	o	o	28
				Dead ova	o	o	o	o	o	o	o	+	1	+	−	+	+	o	3	2	2	2	
3	15 Female	106	105	Live ova	+	+	+	+	+	+	1	+	+	+	o	o	o	o	o	o	o	o	40
				Dead ova	o	o	o	+	+	+	1	+	+	o	o	o	o	2	o	o	o	o	
4	7 Female	53	51	Live ova	+	+	+	+	+	+	+	+	+	+	+	+	+	−	o	o	o	o	23
				Dead ova	o	o	o	o	o	o	o	o	1	o	o	+	+	−	3	1	2	o	
5	9 Male	73	73·5	Live ova	+	+	+	+	+	+	+	+	+	+	+	+	+	o	·1	+	o	o	33
				Dead ova	o	o	o	o	o	+	+	+	1	+	o	o	+	o	2	o	3	o	
6	11 Male	71	69	Live ova	+	−	+	+	+	+	+	+	+	+	+	+	+	o	−	o	o	o	32
				Dead ova	o	−	o	o	+	o	+	+	+	+	+	+	+	−	+	o	o	1	

Table I shows that all six cases had ceased to pass live ova by the twenty-fourth day after the beginning of treatment. As regards general reaction it will be noted that five of the six cases lost from one to three pounds in weight. In one instance (Case No. 1) persistent vomiting, lasting four days, occurred, and it was not considered advisable to continue the course. The repeated intramuscular injection of four to five cubic centimetres of fluid was associated with a considerable degree of local pain, which appeared to be due more to the actual bulk of the fluid injected than to any local reaction caused by the drug.

In addition to these 'Fouadin' cases, one child, aged sixteen, with a severe *S. haematobium* infection was treated with a course of 'Auremetine' by the mouth. The dosage and length of treatment were in accordance with the recommendations of the proprietors, Messrs. Martindale & Co., for the treatment of amoebic dysentery. The results obtained are shown below in Table II.

TABLE II

Showing the results of 'Auremetine' treatment on one West African child severely infected with *S. haematobium*

Age and Sex	Weight in Pounds		Before treatment commenced	Days after the first day of treatment. Last day of treatment shown by double line.																Total dose in grains
	Before Treatment	After Treatment		3	4	5	6	7	8	10	11	12	14	16	18	20	22	24	26	
16 Female	111	114	Live ova +	+	+	+	+	o	+	+	+	+	o	+	o	1	+	b	o	47
			Dead ova o	o	o	o	o	o	+	o	+	o	+	1	+	1	o	o	5	

The results of treatment, in this single case, were similar to those previously recorded with Emetine Periodide (Gordon, 1926), that is to say, live ova disappeared from the urine by the end of the course. Vomiting did not occur at any time and no ill-effects were noted.

II. AFTER-RESULTS OF TREATMENT WITH EMETINE PERIODIDE AND EMETINE HYDROCHLORIDE

One of us (Gordon, 1926) has recorded the results of treating twenty-eight cases of *S. haematobium* infection in native children with Emetine Periodide and with Emetine Hydrochloride, and has shown that both these drugs cause disappearance of live ova from the urine. Our visit to the same district, four years later, has enabled us to re-examine some of these cases. Of the twenty-eight cases originally treated, fourteen were still living in the district; the results of re-examining their urines are shown in Table III.

It will be seen from the Table (p. 447) that the majority of the children treated with Emetine Hydrochloride or with Emetine Periodide have either relapsed or become re-infected. An examination of the remaining seventy untreated children, living in the school, showed that thirty-two (46 per cent.) were passing *S. haematobium* ova in their urine. This observation bears out our previous statement as to the high degree of infection in the district, and renders it impossible to state whether the fourteen children, apparently successfully treated in 1926, but found to be passing live ova in 1930, were cases of relapse or re-infection.

We are much indebted to Miss E. High, Sister-in-charge of the United Brethren in Christ Missionary Hospital at Jiama, for her assistance in carrying out the course of treatment.

<p align="center">TABLE III</p>

Showing the after-results of treatment with Emetine Hydrochloride and with Emetine Periodide on fourteen West African children severely infected with S. *haematobium*.

In this Table each child is given the same number as in the Report published in 1926.

The degree of infection is indicated by the number of + signs.

TREATED WITH EMETINE HYDROCHLORIDE IN 1926						TREATED WITH EMETINE PERIODIDE IN 1926					
Case No.		1926 Examination at end of treatment	1930 Examinations First	Second	Third	Case No.		1926 Examination at end of treatment	1930 Examinations First	Second	Third
1	Live ova	o	o	o	o	1	Live ova	o	+	−	−
	Dead ova	o	o	o	o		Dead ova	2	o	−	−
2	Live ova	o	o	o	o	5	Live ova	o	o	o	o
	Dead ova	o	o	o	o		Dead ova	o	o	o	o
3	Live ova	o	+++	−	−	7	Live ova	o	+	−	−
	Dead ova	4	o	−	−		Dead ova	1	o	−	−
4	Live ova	o	+	−	−	11	Live ova	o	+++	−	−
	Dead ova	+++	o	−	−		Dead ova	o	o	−	−
8	Live ova	o	+++	−	−	12	Live ova	o	+++	−	−
	Dead ova	o	o	−	−		Dead ova	10	o	−	−
11	Live ova	o	o	+	−	13	Live ova	o	+	−	−
	Dead ova	o	+++	++	−		Dead ova	++	o	−	−
12	Live ova	o	+++	−	−						
	Dead ova	2	o	−	−						
13	Live ova	o	o	o	o						
	Dead ova	1	o	o	o						

<p align="center">REFERENCE</p>

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STUDIES IN CHEMOTHERAPY*

III. THE ACTION *IN VITRO* OF CERTAIN ARSENICAL AND ANTIMONIAL COMPOUNDS ON *T. RHODESIENSE* AND ON ATOXYL- AND ACRIFLAVINE-RESISTANT STRAINS OF THIS PARASITE

BY

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AND

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The method of testing the therapeutic action of drugs by injecting them into infected laboratory animals, although an indispensable procedure for obtaining the vital information whether or not a drug is of any practical value for the treatment of the infection, is not entirely adequate if the object of the enquiry be to obtain information regarding the mechanism of the action of the drugs in question. It fails to afford an unequivocal answer to such questions as the following. Does the drug as such act directly on the parasites, or does it become parasiticidal only after it has undergone some change in the body of the host ; or again does the drug act by stimulating the body of the host to produce a parasiticidal substance ? Each of these alternatives has had its advocates, but the facts upon which a final decision could be reached are not yet forthcoming. It was considerations of this nature which led us to the belief that possibly information of value might be obtained from an examination of the trypanocidal power of various drugs *in vitro*, thereby eliminating, in the first instance, all the unknown factors, of uncertain, and possibly inconstant, value, dependent on the vertebrate host. Much work has already been done on this subject, but, as will be seen from the summary which follows, it has for one reason or another little real value.

Ehrlich (1908) observed that whereas a number of trypanocides, e.g., fuchsin, the triphenylmethane dyes, salts of arsenious acid, and tartar-emetic killed the trypanosomes in the test tube, this was not the case with arsanilate and arsacetin. He found, moreover, that although arsanilate and arsacetin, even in 1 to 5 per cent. solutions, had practically no action, a solution of the reduction products, viz. : p-aminophenylarsenoxide and p-diaminoarsenobenzol in dilution of 1 : 100,000 killed the parasites at once, and in a dilution of 1 : 1,000,000 killed them within thirty minutes.

Jacoby and Schütze (1908) stated that although atoxyl in one per cent. solution had only a feeble trypanocidal action, inorganic arsenicals were active even in extreme dilutions, a concentration of only 1 : 200,000 of arsenious acid sufficing rapidly to immobilize the parasites. These conclusions received support from the work of Friedberger (1908), Uhlenhuth (1908), Breinl and Nierenstein (1909), and Neven (1909).

Plimmer and Fry (1909) found that, contrary to the statements of other workers, a solution of 1 : 500 atoxyl immobilized trypanosomes in two hours, and that a solution of 1 : 100 arsenophenylglycine immobilized them within an hour. The drugs were dissolved in physiological saline and mixed with an equal volume of blood containing T. evansi ; these were watched under sealed cover-slip preparations kept at room temperature

Schilling (1909) found that arsenophenylglycine, notwithstanding its powerful therapeutic effect, had a feeble action in vitro, a 10 per cent. solution destroying the parasites only after 50 minutes, whilst a 2 per cent. solution had no effect in this period, at room temperature. Similar observations are recorded by Fusco (1910) and by Zwick and Fischer (1910)

Halberstaedter (1912) examined the trypanocidal action of salvarsan and found that a strain of T. equinum, resistant to arsenicals in vivo, withstood in vitro a concentration of 1 : 2,000, whilst a normal strain of T. brucei was killed within twenty minutes by a 1 : 100,000 solution of the drug.

Gonder (1912) advanced the hypothesis that arsenophenylglycine and salvarsan, although failing to immobilize the trypanosomes in vitro, nevertheless became fixed to them, as parasites which had been in contact with the drugs at room temperature for five minutes

failed to infect mice even though they were actively motile at the time of injection. Very similar observations were made by Castelli (1913) who showed that contact of trypanosomes with solutions of 1 : 2000 to 1 : 8000 of salvarsan for fifteen minutes at 37° C. failed to immobilize the trypanosomes, but that infection did not result when the parasites were subsequently washed and injected into mice. Moldovan (1914) and Kolmer, Schamberg and Raiziss (1917) on the contrary state that salvarsan has a definite trypanocidal action *in vitro*. Adler (1921) recorded that sodium phenylglycineamide arsenate had no appreciable trypanocidal action *in vitro ;* and Dubois (1926) wrote that just as atoxyl is inactive *in vitro*, so sodium *p*-glycineamidephenylarsenate in one per cent. solution did not destroy the motility of trypanosomes even after many hours contact *in vitro*, and that the parasites after washing were still infective for mice.

From this brief summary of some of the principal papers the issue appears somewhat confused. There, however, seems to be a consensus of opinion that the pentavalent arsenicals are inactive *in vitro*, but that their reduced trivalent compounds are exceedingly active. There is, apparently a marked difference of opinion regarding the question whether the arsenobenzols, e.g., arsenophenyl-glycine and salvarsan are active or not.

Practically all this work, however, has for one or more reasons little real value. None of the investigators had succeeded in devising conditions of experiment whereby the enquiry could be satisfactorily conducted. The experiments were almost invariably made at room temperature, and the period of observation limited to an hour or two. These obviously unsatisfactory limitations were undoubtedly imposed by the fact that the conditions of experiment did not suffice to maintain the parasites in the control observations, where no drug was involved, for more than a very short period. The medium in which the drugs were diluted, and the trypanosomes suspended, was usually physiological saline or some modification thereof. As we have shown in a previous paper (1929) trypanosomes will not survive when suspended in such media for more than an hour or so at 37°C.

These facts seem to have been grasped so long ago as 1912 by Rothermundt and Dale, who attributed the failure of previous workers, to demonstrate the trypanocidal action of atoxyl *in vitro*,

to the fact that they had used unsuitable media. Rothermundt and Dale stated that in saline at 37°C. the trypanosomes lost their motility within two hours—a period which they consider to be too short for the action of atoxyl to be manifest—whereas survival of the parasites was prolonged to a period varying from two to four hours at 37°C., and from 8 to 24 hours at 22°C., when they were suspended in defibrinated guinea-pig blood : they subsequently discovered that guinea-pig serum was distinctly superior to defibrinated blood, and that in this medium the parasites remained alive at 37°C. for at least five hours. Rothermundt and Dale found that in such a medium a 1.5 per cent. solution of atoxyl rendered trypanosomes immobile within 2½ hours. Terry (1912 and 1915) likewise emphasizes the advantages of serum as a medium for experiments *in vitro* on the trypanocidal action of drugs ; he shows that a one per cent. solution of atoxyl had no effect on trypanosomes, whereas a 1 : 1,000,000 solution of *p*-aminophenylarsenoxide killed them in thirty minutes.

Papamarku (1927) appears to have been the first to have applied these principles in a systematic attempt to investigate the action of drugs *in vitro*. He employed deactivated rabbit serum as a medium for suspending the trypanosomes and spirochaetes. The blood of heavily infected mice was so diluted with the deactivated rabbit serum that a suspension was obtained showing three to eight parasites to a microscope field. This suspension was then divided equally amongst a number of small tubes and half volumes of various dilutions of different drugs added ; the fluid was then covered with a layer of liquid paraffin. The tubes were stored at laboratory temperature and the contents sampled from time to time by means of a fine capillary tube passed through the layer of liquid paraffin. With this technique Papamarku found that the activity of the parasites in the control tubes was preserved for at least 48 hours. He records that a solution of neosalvarsan in concentration of 1 in 10,000,000 destroyed the trypanosomes within two hours, and 1 in 20,000,000 within 24 hours ; a solution of 1 in 900,000 trypaflavine destroyed the parasites within 24 hours ; a solution of 1 in 900,000 diaminoacridine nitrate killed them within 30 minutes, and 1 in 3,000,000 within 48 hours : a solution of antimony trioxide 1 : 300,000 killed them within 24 hours ; a solution of tartar-emetic 1 : 300,000 killed them within 24 hours, and 1 : 9,000,000 within 48 hours.

This work of Papamarku undoubtedly represents a distinct advance on any previous attempt to examine the trypanocidal action of drugs *in vitro*. It is, however, unfortunate that his observations were conducted at laboratory temperature instead of at 37°C.

In our view the satisfactory determination of the trypanocidal action of drugs *in vitro* necessitated the elaboration of a technique whereby the pathogenic trypanosomes could be maintained alive in undiminished numbers, and, so far as one could ascertain, in a condition of unlowered vitality, for a reasonable period, which we arbitrarily decided to be 24 hours, at 37°C. The technique, which we found to satisfy these conditions, is fully described in a previous paper (1929). In this paper it was shown that, provided the concentration of trypanosomes did not greatly exceed about 1,000 per c.mm., the parasites would remain alive in undiminished numbers, and in a state of unlowered vitality, for at least 24 hours at 37°C. in a medium consisting of equal parts of sheep, or rabbit, serum and Ringer solution containing 0.2 per cent. glucose.

In all the observations recorded in the present paper this medium was used, the rabbit, or sheep, serum being heated to 62°-63°C. for half an hour before the addition of the Ringer-glucose solution. Care was taken so far as possible to avoid bacterial contamination, and the blood of the infected mouse from which the trypanosomes were obtained was so diluted that the concentration of parasites in the nutrient medium was between 1,000 and 1,500 per c.mm. Preliminary experiments immediately showed the importance of temperature in the reaction—all drugs exerting a much more pronounced action at 37°C. than at laboratory temperature— and we consequently decided to limit ourselves to experiments conducted at 37°C. as the data collected under these conditions could much more reasonably be compared with that obtained from animal experiments.

The adoption of this technique appeared to us to have a number of definite advantages. In the first place it enabled the observations to be carried out at body temperature, and for a length of time which is considerably greater than the period required by the drugs employed to sterilize the peripheral blood of infected mice. In the second place the medium is relatively simple and approximates fairly closely to that in which the drugs find themselves when injected

into the living animal It is, of course, possible that the action of certain drugs may to some extent be dependent on the nature of the medium in which they are dissolved, and indeed there are in the literature certain statements which suggest that this is the case. Schilling and Goretti (1914) assert that tartar-emetic has *in vitro* no action on *T. brucei* when it is dissolved in citrate bouillon, but that it is powerfully active when dissolved in horse serum—1 : 9,000 killing the parasites in 45 minutes ; they add, however, that with salvarsan, atoxyl and trypanred the effect was the same whether the medium was citrate bouillon or horse serum. On the other hand Reiner and Leonard (1930) write that ' if an emulsion of trypanosomes was brought in contact with neoarsphenamine dissolved in a dilution of 1 : 5,000, or with sodium atoxylate, and allowed to stand for 20 minutes, then centrifuged and washed, the trypanosomes remained almost as virulent as the controls. Time of action and dilution of the chemotherapeutic agent were chosen far below the limits which produced a damaging effect as shown by the change in motility.' With regard to neoarsphenamine they add that ' the active compound is probably a loose combination of serum protein (globulin) and neoarsphenamine, for it is also formed *in vitro* by mixing neoarsphenamine with serum or plasma. Atoxyl and tryparsamide are, however, as little active with serum as either substance in broth.' There is thus so far as salvarsan and neosalvarsan are concerned, a possible discrepancy between Schilling, and Reiner and Leonard, the former saying that salvarsan is equally trypanocidal *in vitro* whether diluted with serum or broth, and the latter that neosalvarsan is active in serum but inactive in broth. Probably an unsatisfactory technique, and the difficulty of keeping trypanosomes alive in broth at 37°C. for any length of time account for these apparent differences. We have, however, not concerned ourselves with matters of this nature. If it be true that certain drugs behave differently according to whether they are dissolved in serum or in other fluids, it merely emphasizes the importance of employing serum as the basis for the medium in any work which has as its main object the comparison of the action of drugs *in vitro* and *in vivo*..

In our first experiments the drugs used were the pentavalent arsenical compounds, atoxyl and tryparsamide, and the trivalent

compounds, p-aminophenylarsenoxide (reduced atoxyl), and halarsol. Later a number of other organic preparations of arsenic* and of antimony were used as well as sodium arsenite, tartar emetic, acriflavine, phenol and aniline. For convenience of reference the structural formula of each of the compounds used is given (p. 468). The trypanosome strains employed were *T. rhodesiense*† and atoxyl- and acriflavine- resistant varieties of this strain, prepared by treating infected mice with subcurative doses of atoxyl and acriflavine respectively, and gradually increasing the doses as relapses occurred in the same animals and in animals of later passage : by this means strains were produced which were completely resistant to the maximum tolerated dose of the drug employed.

EXPERIMENT. A rabbit was bled from the heart and the blood allowed to clot for two hours at 37°C. ; the serum was then removed from the clot, and after centrifuging at high speed to remove all cellular material and débris, was heated at 62°-63°C. for 30 minutes. To each of Tubes 1, 10, 17, 25, 33, 39, 45, 52, 60, 66, 72 and 79 was added 0·5 c.c. of the serum. The rest of the serum was then diluted with an equal volume of Ringer-glucose solution, and 0·5 c.c. of the diluted serum added to each of the remaining tubes shown in Table I. Dilutions of the four drugs to be examined were then made in Ringer-glucose solution as follows :— atoxyl 1 : 50, tryparsamide 1 : 50, p-aminophenylarsenoxide 1 : 6,400,000, and halarsol 1 : 6,400,000. The various concentrations of atoxyl in the nutrient medium shown in the table were made by adding 0·5 c.c. of the 1 : 50 atoxyl solution to Tube 1 and then, after mixing thoroughly, transferring 0·5 c.c. from this tube to the next and so on to Tube 9, from which 0·5 c.c. was then thrown away. By this means it was contrived that the concentration of drug in any tube was half that in the previous tube. The various concentrations of the other drugs were prepared in the same manner.

The trypanocidal action of the various drugs in respect of each of the three strains of *T. rhodesiense* (normal, atoxyl- and acriflavine-resistant) was then examined by adding 0·025 c.c. of a suspension of trypanosomes obtained by suitably diluting the blood of a heavily infected mouse with Ringer-glucose solution, as described in our previous paper (1929) ; the tubes, after being covered with glass caps, were placed in the water bath at 37°C., and the contents examined at stated intervals.

The figures contained in Table I show in the most graphic manner that whereas the pentavalent arsenicals—atoxyl and tryparsamide— are but slightly trypanocidal *in vitro*, the trivalent compounds— p-aminophenylarsenoxide and halarsol—are extraordinarily active.‡

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† This strain is the one referred to in our previous papers (1929 and 1930) ; it was isolated from man in 1923, and since maintained by passage through mice.

‡ The changes produced in the trypanosomes by the action of the various drugs were similar to those described in our previous paper on the action of normal human serum ; the parasites underwent various degenerative changes and finally all trace of them disappeared. .

As will be seen, with all the drugs the trypanocidal effect varied considerably with the duration of the reaction, thus atoxyl 1 : 200 killed the normal trypanosome strain in 4 hours, 1 : 800 in 8 hours, 1 : 1,600 in 12 hours and 1 : 6,400 in 24 hours ; tryparsamide was even less active. *P*-aminophenylarsenoxide 1 : 51,200,000 killed the normal trypanosome strain in 4 hours, 1 : 102,400,000 in 8 hours, 1 : 409,600,000 in 12 hours after which there was no further change ; halarsol was almost equally active. From these figures it appears that the pentavalent atoxyl is, according to the length of time of the reaction, only about $\dfrac{1}{256,000}$ to $\dfrac{1}{64,000}$ times as active as its reduction product, viz., the trivalent *p*-aminophenylarsenoxide.

TABLE I.

Showing the trypanocidal action of various organic preparations of arsenic on trypanosomes (normal, atoxyl-resistant and acriflavine-resistant strains), suspended in Rabbit serum-Ringer-glucose solution, at 37°C.

Tube	Drug	Concentration of drug in nutrient medium.	Strain	No. of living trypanosomes per 256 squares of the hæmocytometer scale							
				Start	2 hours	4 hours	8 hours	12 hours	16 hours	20 hours	24 hours
1		1 : 100			45	0	...	...	...	...	...
2		1 : 200			...	4	...	...	...	...	...
3		1 : 400			...	35	0	...	...	...	...
4		1 : 800			...	86	3	...	...	...	...
5	Atoxyl	1 : 1,600			...	...	39	0	...	...	...
6		1 : 3,200			...	...	104	60	0	...	...
7		1 : 6,400			...	...	...	88	50	30	0
8		1 : 12,800			...	...	...	...	80	82	60
9		1 : 25,600			...	...	...	...	...	...	80
10		1 : 100			46	24	...	...	...	...	...
11		1 : 200			...	62	0	...	...	...	...
12		1 : 400			...	...	37	0	...	...	...
13	Tryparsamide	1 : 800			...	...	66	53	52	0	...
14		1 : 1,600			...	...	...	81	51	0	...
15		1 : 3,200			...	...	...	...	71	68	74
16		1 : 6,400			...	...	...	...	...	...	90
17		1 : 12,800,000 ...		66	0	...	...	...	...	...	...
18		1 : 25,600,000 ...			6	0	...	...	...	...	...
19		1 : 51,200,000 ...			32	0	...	...	...	...	...
20	*P*-aminophenyl-	1 : 102,400,000 ...			52	36	0	...	...	...	...
21	arsenoxide	1 : 204,800,000 ...			...	52	21	0	...	...	...
22		1 : 409,600,000 ...			...	...	78	1	0	...	...
23		1 : 819,200,000 ...			...	...	...	104	84	76	82
24		1 : 1,638,400,000 ...			...	...	...	...	86	90	90
25		1 : 12,800,000 ...			0	...	...	...	...	...	...
26		1 : 25,600,000 ...			32	0	...	...	...	...	...
27		1 : 51,200,000 ...			55	21	0	...	...	...	...
28	Halarsol	1 : 102,400,000 ...			...	66	5	0	...	...	...
29		1 : 204,800,000 ...			...	...	79	12	0	...	...
30		1 : 409,600,000 ...			...	...	...	100	88	96	88
31		1 : 819,200,000 ...			...	...	...	...	...	...	108
32		Control			...	...	...	...	92	96	90

Normal strain

TABLE I—*continued*.

Tube	Drug	Concentration of drug in nutrient medium	Strain	Start	2 hours	4 hours	8 hours	12 hours	16 hours	20 hours	24 hours
33		1 : 100			82	75	0	...	...	...	...
34		1 : 200			...	84	0	...	...	...	...
35	Atoxyl	1: 400			...	...	20	0	...	...	...
36		1 : 800			...	...	128	41	0	...	...
37		1 : 1,600			...	...	...	94	66	31	13
38		1 : 3,200			...	...	...	...	104	...	102
39		1 : 100			88	26	0	...	...	...	...
40		1 : 200			...	106	0	...	...	...	...
41	Tryparsamide	1: 400			...	...	56	0	...	...	...
42		1 : 800	Atoxyl-resistant strain		...	...	118	13	0	...	...
43		1 : 1,600			...	...	...	111	114	84	35
44		1 : 3,200			...	...	...	...	...	...	104
45		1 : 3,200,000 ...			0	0	...	...	...	...	...
46		1 : 6,400,000 ...		90	5	0	...	...	...	...	...
47		1 : 12,800,000 ...			77	6	0	...	...	...	...
48	P-aminophenyl	1 : 25,600,000 ...			...	112	0	...	...	...	...
49	arsenoxide ...	1 : 51,200,000 ...			...	...	78	11	0	...	...
50		1: 102,400,000 ...			...	...	108	98	84	8	0
51		1 : 204,800,000 ...			...	...	...	...	...	134	128
52		1 : 100,000 ...			0	...	...	...	...	...	...
53		1 : 200,000 ...			9	0	...	...	...	...	...
54		1 : 400,000 ...			94	2	0	...	...	...	...
55	Halarsol	1 : 800,000 ...			...	61	0	...	...	...	...
56		1 : 1,600,000 ...			...	77	27	0	...	...	...
57		1 : 3,200,000 ...			...	...	56	60	23	2	0
58		1 : 6,400,000 ...			...	...	102	118	114	104	86
59		Control			...	...	...	104	...	...	128
60		1 : 100			66	23	0	...	...	...	...
61		1 : 200			...	67	0	...	...	...	...
62	Atoxyl	1 : 400			...	...	40	0	...	...	...
63		1 : 800			...	...	42	15	0	...	...
64		1 : 1,600			...	...	78	33	12	0	...
65		1 : 3,200			...	...	...	76	80	80	78
66		1 : 100			69	12	0	...	...	...	...
67		1 : 200			...	63	0	...	...	...	...
68	Tryparsamide	1 : 400			...	...	40	0	...	...	...
69		1 : 800			...	...	...	9	0	...	...
70		1 : 1,600			...	...	91	74	46	38	18
71		1 : 3,200	Acriflavine-resistant strain		...	...	...	100	102	...	75
72		1 : 3,200,000 ...			3	0	...	...	...	...	...
73		1 : 6,400,000 ...			9	0	...	...	...	...	...
74	P-aminophenyl	1 : 12,800,000 ...		70	69	2	0	...	...	...	...
75	arsenoxide ...	1 : 25,600,000 ...			...	70	.0	...	...	...	...
76		1 : 51,200,000 ...			...	84	55	1	...	...	...
77		1 : 102,400,000 ...			...	...	64	53	46	0	...
78		1 : 204,800,000 ...			...	...	...	68	69	66	69
79		1 : 100,000 ...			0	...	...	...	...	...	...
80		1 : 200,000 ...			49	0	...	...	...	...	...
81		1 : 400,000 ...			59	24	0	...	...	...	...
82	Halarsol	1 : 800,000 ...			73	57	1	...	...	...	...
83		1 : 1,600,000 ...			...	78	44	0	...	...	...
84		1 : 3,200,000 ...			...	...	79	78	12	0	...
85		1 : 6,400,000 ...			...	...	...	93	68	41	5
86		1 : 12,800,000 ...			...	...	...	...	74	...	88
87		Control			...	...	...	...	...	...	73

In the case of the atoxyl-resistant strain atoxyl 1 : 100 failed to kill the trypanosomes in 4 hours, 1 : 200 killed them in 8 hours, 1 : 400 in 12 hours and practically 1 : 1,600 in 24 hours ; tryparsamide behaved in very much the same manner. *P*-aminophenylarsenoxide 1 : 12,800,000 killed the trypanosomes in 4 hours, 1 : 25,600,000 in 8 hours, practically 1 : 51,200,000 in 12 hours and 1 : 102,400,000 in 24 hours ; but curiously enough in the case of halarsol, although its trypanocidal effect on the normal strain was almost equal to that of *p*-aminophenylarsenoxide, its action on the atoxyl-resistant strain was very much less, e.g., 1 : 400,000 killed the trypanosomes in 4 hours, 1 : 800,000 in 8 hours, 1 : 1,600,000 in 12 hours and 1 : 3,200,000 in 24 hours. The acriflavine-resistant strain reacted to all four drugs in exactly the same way as did the atoxyl-resistant strain.

Summarizing the information obtained from experiments of this nature we find :—

1. That the pentavalent arsenicals, atoxyl and tryparsamide, are relatively but slightly trypanocidal *in vitro* at 37°C.

2. The trivalent arsenicals, *p*-aminophenylarsenoxide and halarsol are exceedingly trypanocidal *in vitro* at 37°C., killing the parasites even when diluted several hundred million times.

3. The atoxyl-resistant strain is, in comparison with the normal strain, definitely resistant *in vitro* to all four drugs. With the pentavalent compounds, atoxyl and tryparsamide, this relative resistance is but slight, and this is likewise so with the trivalent *p*-aminophenylarsenoxide, but in the case of the trivalent halarsol the resistance is much greater. Accepting the figures at their face value, it appears that the atoxyl resistant strain is capable of withstanding *in vitro* about twice the concentration of tryparsamide as is the normal strain, about 4 times that of atoxyl or of *p*-aminophenyl-arsenoxide, and about 64 times that of halarsol.

4. The acriflavine-resistant strain proved exactly as resistant to all four arsenicals as did the atoxyl-resistant strain.

There are thus several interesting points which emerge from this work. Firstly, the trypanocidal activity *in vitro* of the trivalent compounds is such that it is quite unnecessary to assume that their therapeutic effect *in vivo* is dependent on anything other than the direct action of the unchanged drugs on the parasites ; this, however,

cannot be said of the relatively inert pentavalent compounds. Secondly, the character of drug resistance is inherent in the parasites themselves and is not, as has often been assumed, dependent on the host. Thirdly, a strain made resistant to acriflavine is just as resistant to organic arsenicals as is the atoxyl-resistant strain itself. Fourthly, the degree of resistance varies greatly with different drugs, i.e., with atoxyl, tryparsamide, and p-aminophenylarsenoxide the resistant strains are only about 4 times as resistant as is the normal strain, whereas with halarsol the resistant strain is about 64 times as resistant as is the normal strain.

These facts appeared to us to be so interesting that we decided to elaborate the investigation with a view to discovering whether it was possible to obtain definite quantitative values for the normal and resistant strains in respect of the whole series of drugs referred to above. Unfortunately, we were soon faced with the disconcerting fact that the same drug did not exhibit the same trypanocidal power on every occasion. For example, with halarsol we found that although the dilutest solution necessary to kill all the trypanosomes of the normal strain within a period of 24 hours was, as a rule, from 1 : 102,400,000 to 1 : 409,600,000 it varied, in extreme cases, from 1 : 25,600,000 to 1 : 819,200,000 It therefore soon became evident that if we were to proceed with this investigation it would first of all be necessary to discover the cause of this variation.

The most promising field for search appeared to be the nutrient medium. In every experiment this consisted of equal parts of either rabbit, or sheep, serum, carefully separated from all cellular material by centrifugation at high speed, and heated to 62°-63°C. for half an hour, and of Ringer-glucose solution. In the long series of experiments the sera of many rabbits and sheep were employed. Sometimes the serum was obtained on the day of the experiment and sometimes one or two days previously. Sometimes the drugs were added to the nutrient medium immediately before the trypanosomes, but on other occasions—especially when a large experiment involving the testing of many drugs was contemplated—the drugs were added to the nutrient medium one or even two days before the commencement of the experiment, the mixture of drugs and medium being stored in the ice-box during the interval.

We, therefore, set ourselves to enquire whether the individual

rabbit or sheep serum was responsible for the different results, and also whether storing the mixture of drugs and nutrient medium in the ice-box for a day or two before the addition of the trypanosomes and commencement of the experiment, gave different values from freshly-mixed medium and drug. The results of many experiments showed that the sera of different rabbits gave slightly different values, as did also those of different sheep ; and, furthermore, that as a general rule the drugs showed a higher trypanocidal reading when diluted in nutrient media made from rabbit serum than in those made from sheep serum.

The experiments made with the object of ascertaining the effect of leaving the drug in contact with the nutrient medium, or of merely storing the nutrient medium in the absence of drug, for some days at low temperature (5° C.), gave strangely divergent results. On certain occasions neither of these procedures had any apparent effect, but on other occasions they exerted a markedly inhibitory effect on the trypanocidal action of the drug. This is clearly shown in the experiment recorded in Table II.

EXPERIMENT. Two rabbits, B and D, were bled from the heart and the blood allowed to clot for two hours at 37° C. The sera were then separated from the clots, and, after centrifuging at high speed to remove all cellular material and débris, were heated at 62°-63° C. for thirty minutes. From these sera, nutrient media were prepared in the usual way, and the trypanocidal titre of halarsol, for the normal strain, determined with each medium under the following conditions :—

1. Freshly-prepared medium mixed with freshly-prepared solution of halarsol immediately before the addition of the trypanosomes.

2. Freshly-prepared medium mixed with a six-day-old solution of halarsol immediately before the addition of trypanosomes.

3. Freshly-prepared medium mixed with freshly-prepared solution of halarsol, and the mixture stored at 5° C. for 48 hours, before the addition of the trypanosomes.

4. Medium and halarsol solution stored separately at 5° C. for 48 hours before mixing and the addition of the trypanosomes.

The details of technique were those described in the previous experiment.

The figures set forth in Table II show that when the trypanosomes were added immediately after the mixture of freshly-prepared medium and freshly-prepared drug there was but little difference whether the medium was made with the serum of Rabbit B or Rabbit D. In the former case (Rabbit B) the trypanocidal titre of halarsol was in twenty-four hours almost 1 : 409,600,000, and in the

latter case (Rabbit D) nearly 1 : 819,200,000. But slightly lower values were obtained when the trypanosomes were added immediately after the mixture of the fresh medium and halarsol solution kept for six days at 5° C. When, however, the parasites were added to the mixture of medium and drug which had stood for two days at 5° C., the difference in the trypanocidal titre obtained with the two media was very pronounced. With the medium made from the serum of Rabbit B the trypanocidal titre of halarsol was less than 1 : 51,200,000, but with the media made from the serum of Rabbit D it was practically unchanged at nearly 1 : 819,200,000. A similar but rather less marked divergence was observed in the case when the nutrient media and drug were kept separately at 5° C. for two days and then mixed immediately before the addition of the trypanosomes.

From experiments of this nature it is clear that leaving the drug in contact with the nutrient medium at low temperatures for 24 to 48 hours suffices in certain cases to modify greatly the trypanocidal titre obtained for the drug, and a similar though somewhat smaller fall in the titre may be produced by merely storing the medium at 5° C. for a like period before the addition of the drug. Why this should be so, and particularly why it should be so in the case of certain sera only, we are unable to say. A possible explanation seems to be that the individual sera differ slightly, and that certain of them contain particles in colloidal suspension which are capable of adsorbing minute quantities of the drug, so that when the latter is present in such infinitely minute amount as one part in several hundred millions an appreciable proportion of what is present is adsorbed, and consequently the amount left available to act on the trypanosomes is proportionately reduced with the result that the trypanocidal value is apparently considerably lowered.

As a result of this work we reached the conclusion that the serum used in the nutrient medium is an important factor in determining the exact trypanocidal value of a drug. Apparently the freshly-deactivated sera of different rabbits and of different sheep vary slightly and as a general rule higher values are obtained with media made from rabbit serum. A much more pronounced cause of variation, however, is the length of time the drug and nutrient

TABLE II

Showing the inhibition of the trypanocidal action of halarsol sometimes encountered when the drug is left in contact with the nutrient medium at 5° C. for 48 hours, and even when the nutrient medium alone is stored at 5° C. for a similar period.

Tube	Source of serum used in nutrient medium	Conditions of experiment as regards length of time nutrient medium kept at 5° C., either alone or after addition of drug	Concentration of halarsol in nutrient medium	Number of living trypanosomes per 256 squares of the haemocytometer scale				
				Start	4 hours	8 hours	12 hours	24 hours
1	Rabbit B	Freshly-prepared medium mixed with freshly-prepared solution of halarsol immediately before addition of trypanosomes.	1 : 6,400,000		...	...	...	...
2			1 : 12,800,000		0	...	...	...
3			1 : 25,600,000		0	...	...	...
4			1 : 51,200,000		3	...	...	...
5			1 : 102,400,000		41	0	...	...
6			1 : 204,800,000		122	61	4	0
7			1 : 409,600,000		...	134	114	5
8			1 : 819,200,000		...	...	...	71
9			1 : 1,638,400,000		...	...	...	92
10			1 : 3,276,800,000		...	...	...	90
11	Rabbit D		1 : 6,400,000		...	...	...	...
12			1 : 12,800,000		...	...	...	...
13			1 : 25,600,000		0	...	...	...
14			1 : 51,200,000		2	...	...	...
15			1 : 102,400,000		15	0	...	...
16			1 : 204,800,000		50	15	0	...
17			1 : 409,600,000		84	63	34	0
18			1 : 819,200,000		...	...	50	17
19			1 : 1,638,400,000		...	...	66	60
20			1 : 3,276,800,000		...	...	62	80
21	Rabbit B	Freshly-prepared medium mixed with six-day-old solution of halarsol immediately before addition of trypanosomes.	1 : 6,400,000	76	...	...	...	...
22			1 : 12,800,000		...	...	...	...
23			1 : 25,600,000		0	...	...	...
24			1 : 51,200,000		8	0	...	...
25			1 : 102,400,000		84	2	0	...
26			1 : 204,800,000		...	86	13	0
27			1 : 409,600,000		...	...	...	88
28			1 : 819,200,000		...	...	...	134
29			1 : 1,638,400,000		...	...	...	...
30			1 : 3,276,800,000		...	...	...	...
31	Rabbit D		1 : 6,400,000		...	...	...	...
32			1 : 12,800,000		...	...	...	...
33			1 : 25,600,000		...	...	...	...
34			1 : 51,200,000		6	0	...	...
35			1 : 102,400,000		46	6	0	...
36			1 : 204,800,000		64	88	19	0
37			1 : 409,600,000		...	...	45	2
38			1 : 819,200,000		...	...	...	77
39			1 : 1,638,400,000		...	...	...	84
40			1 : 3,276,800,000		...	...	88	44
41		Control, Rabbit B			...	...	66	74
42		Control, Rabbit D			...	...	68	56

TABLE II—continued.

Tube	Source of serum used in nutrient medium	Conditions of experiment as regards length of time nutrient medium kept at 5° C., either alone or after addition of drug	Concentration of halarsol in nutrient medium	Number of living trypanosomes per 256 squares of the haemocytometer scale				
				Start	4 hours	8 hours	12 hours	24 hours
43			1 : 6,400,000		0	...	...	...
44			1 : 12,800,000		61	0	...	...
45			1 : 25,600,000		84	80	0	...
46			1 : 51,200,000		...	...	82	34
47	Rabbit B		1 : 102,400,000		...	...	...	110
48			1 : 204,800,000		...	...	...	...
49			1 : 409,600,000		...	...	...	...
50			1 : 819,200,000		...	...	...	...
51		Freshly-prepared medium mixed with freshly-prepared solution of halarsol and mixture stored at 5° C., for 48 hours before addition of trypanosomes.	1 : 1,638,400,000		...	...	...	...
52			1 : 3,276,800,000		...	...	...	...
53			1 : 6,400,000		0	...	...	...
54			1 : 12,800,000		0	...	...	...
55			1 : 25,600,000		0	...	...	...
56			1: 51,200,000		0	...	...	...
57	Rabbit D		1 : 102,400,000		29	0	...	...
58			1 : 204,800,000		61	11	0	...
59			1 : 409,600,000		...	51	35	0
60			1 : 819,200,000		...	...	46	17
61			1 : 1,638,400,000		...	...	65	61
62			1 : 3,276,800,000	96	...	...	78	74
63			1 : 6,400,000		0	...	...	...
64			1 : 12,800,000,000		0	...	...	...
65			1 : 25,600,000		0	...	...	...
66			1 : 51,200,000		46	0	...	...
67	Rabbit B		1 : 102,400,000		84	72	25	0
68			1 : 204,800,000		...	...	84	76
69			1 : 409,600,000		...	...	...	84
70			1 : 819,200,000		...	...	...	96
71		Medium and halarsol solution stored separately at 5° C., for 48 hours before mixing and addition of trypanosomes.	1 : 1,638,400,000		...	...	...	100
72			1 : 3,276,800,000		...	...	...	98
73			1 : 6,400,000		0	...	...	...
74			1 : 12,800,000		0	...	...	...
75			1 : 25,600,000		0	...	...	...
76			1 : 51,200,000		0	...	...	...
77	Rabbit D		1 : 102,400,000		16	0	...	...
78			1 : 204,800,000		41	7	1	...
79			1 : 409,600,000		43	48	12	0
80			1 : 819,200,000		60	...	62	43
81			1 : 1,638,400,000		...	...	80	90
82			1 : 3,276,800,000		...	...	...	92
83		Control, Rabbit B			...	...	92	90
84		Control, Rabbit D			...	...	88	82

medium have been in contact at low temperatures before the addition of the trypanosomes. In the case of media made from certain sera contact with the drug for 24 to 48 hours suffices greatly to reduce the apparent trypanocidal value of the drug, whereas in the case of media made from other sera the reduction is but slight or even inappreciable.

In view of this it seemed clear that, if we wished to obtain comparable results in the case of a large number of drugs with different strains (normal and drug resistant) of trypanosomes, it was advisable to submit to the following conditions :

1. That all the drugs to be compared should be examined with the same nutrient medium.

2. That the serum used in the medium should be freshly obtained.

3. That the trypanosomes should be added, and the experiment commenced, immediately after the dilution of the drugs in the nutrient medium.

As the trypanocidal action of twenty-one drugs had to be examined on three different strains (normal, atoxyl-resistant and acriflavine-resistant) of trypanosomes nearly 200 c.c. of the serum were required for each experiment ; and consequently it was necessary to employ sheep instead of rabbits for such comparative work. The results of a number of experiments of this kind were found to agree very closely, and although, as already mentioned, the trypanocidal values of most of the active trivalent arsenicals were found to be rather less when tested with media made from sheep serum than with those made from rabbit serum, elaborate experiments, wherein the trypanocidal values of all the drugs were examined at the same time with the same nutrient medium, had the great advantage of enabling the action of one drug to be contrasted with that of another. In Table III we have summarized the trypanocidal action of all the twenty-one drugs on the normal, atoxyl-resistant and acriflavine-resistant strains of *T. rhodesiense.* The experiments were conducted at 37° C. with observations made six hours and twenty-four hours respectively after the addition of the trypanosomes to the medium containing the various dilutions of drugs.

The chief points which emerge from these experiments, appear,

TABLE III

Showing the minimum concentration of drug necessary to destroy within 6 and 24 hours respectively the various strains (normal and resistant) of *T. rhodesiense* suspended in Sheep-serum-Ringer-glucose *in vitro* at 37° C.

Drug	MINIMUM LETHAL CONCENTRATION OF DRUG						Approximate resistant factors
	Normal Strain		Atoxyl-resistant Strain		Acriflavine-resistant Strain		
	Within 6 hours	Within 24 hours	Within 6 hours	Within 24 hours	Within 6 hours	Within 24 hours	
Atoxyl	1 : 200	1 : 1,600	1 : <100	1 : 400	1 : <100	1 : 400	2–4
Arsacetin	1 : 200	1 : 1,600	1 : 100	1 : 800	1 : 100	1 : 800	2
Tryparsamide	1 : 200	1 : 1,600	1 : 100	1 : 1,600	1 : 100	1 : 1,600	1–2
Stovarsol	1 : 400	1 : 1,600	1 : 200/400	1 : 800/1,600	1 : 200/400	1 : 800/1,600	1–2
Halarsol...	1 : 25,600,000	1 : 102,400,000	1 : 800,000	1 : 3,200,000	1 : 800,000	1 : 3,200,000	32
Halarsol thioglycollate	1 : 25,600,000	1 : 102,400,000	1 : 800,000	1 : 3,200,000	1 : 800,000	1 : 3,200,000	32
P-aminophenyl arsenoxide	1 : 51,200,000	1 : 102,400,000	1 : 6,400,000	1 : 25,600,000	1 : 6,400,000	1 : 25,600,000	4–8
Reduced atoxyl thioglycollate	1 : 51,200,000	1 : 102,400,000	1 : 6,400,000	1 : 25,600,000	1 : 6,400,000	1 : 25,600,000	4–8
Reduced arsacetin ...	1 : 25,600,000	1 : 102,400,000	1 : 800,000	1 : 3,200,000	1 : 800,000	1 : 3,200,000	32
Reduced tryparsamide thioglycollate ...	1 : 51,200,000	1 : 204,800,000	1 : 50,000	1 : 200,000	1 : 50,000	1 : 200,000	1024
Reduced stovarsol thioglycollate	1 : 800,000	1 : 6,400,000	1 : 200,000	1 : 400,000	1 : 200,000	1 : 400,000	8–16
Novarsenobillon ...	1 : 12,800,000	1 : 51,200,000	1 : 200,000	1 : 1,600,000	1 : 200,000	1 : 1,600,000	32–64
Arsenophenylglycine ...	1 : 400,000	1 : 3,200,000	1 : 200,000	1 : 1,600,000	1 : 200,000	1 : 1,600,000	2
Arsenophenylglycine-amide ...	1 : 25,600,000	1 : 102,400,000	—	1 : 400,000	—	1 : 400,000	256*
Sodium arsenite ...	1 : 1,600,000	1 : 3,200,000	1 : 1,600,000	1 : 3,200,000	1 : 1,600,000	1 : 3,200,000	1
Stibenyl...	1 : 3,200	1 : 12,800	1 : 3,200	1 : 12,800	1 : 3,200	1 : 12,800	1
Stibosan	1 : 3,200	1 : 12,800	1 : 3,200	1 : 12,800	1 : 3,200	1 : 12,800	1
Potassium antimony tartrate ...	1 : 3,200,000	1 : 6,400,000	1 : 3,200,000	1 : 6,400,000	1 : 3,200,000	1 : 6,400,000	1
Phenol	1 : 800	1 : 3,200	1 : 800	1 : 3,200	1 : 800	1 : 3,200	1
Aniline	1 : 400	1 : 12,800	1 : 400	1 : 6,400	1 : 400	1 : 6,400	1–2
Acriflavine	1 : 1,600,000	1 : 25,600,000	1 : 200,000	1 : 1,600,000	1 : 200,000	1 : 6,000,000	8–16

* Owing to the fact that arsenophenylglycineamide is exceedingly insoluble the figures given for this drug are of doubtful accuracy.

so far as the normal strain of trypanosomes is concerned, to be as follows :

1. The pentavalent arsenical compounds—atoxyl, arsacetin, tryparsamide, and stovarsol—are practically without trypanocidal action, a solution of 1 : 1600 being required to destroy all the parasites within twenty-four hours.

2. Aniline and phenol themselves are several times more active than the pentavalent arsenicals.

3. The pentavalent antimonial compounds are likewise several times more active than the corresponding arsenical compounds, but possibly this may in some measure be due to decomposition of these products which are notably less stable than the arsenical compounds.

4. The trivalent arsenical compounds—p-aminophenylarsenoxide, reduced atoxyl thioglycollate, reduced arsacetin, halarsol, halarsol thioglycollate and reduced tryparsamide thioglycollate—are extraordinarily trypanocidal, such enormous dilutions as 1 : 102,400,000 to 1 : 204,800,000 sufficing to destroy all the trypanosomes within twenty-four hours. From this it appears that the trivalent arsenical compounds are about 100,000 times as trypanocidal as their corresponding pentavalent compounds.

5. Reduced stovarsol thioglycollate proved to be much less active than the other trivalent arsenical compounds examined, 1 : 6,400,000 being required to destroy the parasites within twenty-four hours. It is thus about 4,000 times as active as its corresponding pentavalent compound.

6. Of the arsenobenzols, novarsenobillon—whether previously oxidized or not—proved to be exceedingly active, a dilution of 1 : 51,200,000 being sufficient to destroy the trypanosomes within twenty-four hours. Arsenophenylglycine was likewise active but much less so than N.A.B., the trypanocidal titre being 1 : 3,200,000 ; it is, however, interesting to note that the corresponding amide—arsenophenylglycineamide—was remarkably active, the trypanocidal value being probably about 1 : 102,400,000.*

7. Sodium arsenite and tartar-emetic also displayed considerable activity, the corresponding trypanocidal values being respectively 1 : 3,200,000 and 1 : 6,400,000.

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* Owing to the fact that arsenophenylglycineamide is exceedingly insoluble the figures given for this drug are of doubtful accuracy.

The action of these various drugs on the two resistant strains proved to be no less interesting. The outstanding points appear to be as follows :—

1. The atoxyl-resistant and acriflavine-resistant strains proved to be equally sensitive to the action of the various drugs.

2. To the pentavalent arsenicals, and to aniline, the resistant strains were but slightly more resistant than the normal strain ; as a rule they withstood about twice the concentration of the drugs as did the normal strain.

3. To the various trivalent arsenicals the resistant strains exhibited very different degrees of resistance. In the case of reduced atoxyl-thioglycollate and p-aminophenylarsenoxide the resistant strains withstood about four to eight times, in that of reduced stovarsol thioglycollate about eight to sixteen times, in that of halarsol, halarsol thioglycollate and reduced arsacetin about thirty-two to sixty-four times, and in that of reduced tryparsamide about 1,024 times the concentration which sufficed to destroy the normal strain.

4. To the various arsenobenzols the resistant strains likewise exhibited very different degrees of resistance. To novarsenobillon they withstood thirty-two to sixty-four times, to arsenophenylglycine two to four times, and to arsenophenylglycineamide about 256 times the concentration which proved fatal to the normal strain.

5. To sodium arsenite and to tartar-emetic the resistant strains proved just as susceptible as did the normal strain.

6. To stibosan and stibenyl the resistant strains were likewise nearly as susceptible as the normal strain.

From these facts it appears to us possible to draw certain conclusions of importance. The trypanocidal power of the pentavalent arsenicals *in vitro* is so slight that the therapeutic value of these drugs is in no way dependent upon their direct action ; they must owe their curative value either to the fact that they are reduced in the body of the host to their corresponding trivalent forms, or else to some remarkable capacity of stimulating the body of the host to produce some hitherto unknown trypanocidal substance. The astonishing activity of the trivalent arsenicals, or at least of such of them as have been shown to be of any value in the treatment of trypanosomiasis, appears to warrant the conclusion that their

$$\underset{\text{Atoxyl}}{\overset{\overset{\displaystyle O}{\underset{\displaystyle ||}{}}}{HO-As-ONa}}$$

Atoxyl

NH$_2$

$$\overset{\overset{\displaystyle O}{\underset{\displaystyle ||}{}}}{As}$$
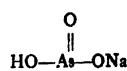
Reduced atoxyl
(p-aminophenylarsenoxide)

NH$_2$

$$As\begin{cases} S.CH_2.COONa \\ S.CH_2.COOH \end{cases}$$

Reduced atoxyl
thioglycollate

NH$_2$

$$\overset{\overset{\displaystyle O}{\underset{\displaystyle ||}{}}}{HO-As-Na}$$

Arsacetin

NH.CO.CH$_3$

$$\overset{\overset{\displaystyle O}{\underset{\displaystyle ||}{}}}{As}$$

Reduced
Arsacetin

NH.CO.CH$_3$

$$\overset{\overset{\displaystyle O}{\underset{\displaystyle ||}{}}}{HO-As-ONa}$$

Tryparsamide

NH.CH$_2$.CONH$_2$

$$As\begin{cases} S.CH_2\ COONa \\ S.CH_2.COOH \end{cases}$$

Reduced
tryparsamide
thioglycollate

NH.CH$_2$.CONH$_2$

$$\overset{\overset{\displaystyle O}{\underset{\displaystyle ||}{}}}{HO-As-ONa}$$

Stovarsol

NH.CO.CH$_3$

OH

$$As\begin{cases} S.CH_2.COONa \\ S.CH_2.COOH \end{cases}$$

Reduced stovarsol
thioglycollate

NH.CO.CH$_3$

OH

AsCl$_2$

Halarsol

NH

OH

$$As\begin{cases} S.CH_2.COONa \\ S.CH_2.COOH \end{cases}$$

Halarsol
thioglycollate

NH$_2$

OH

As ═ As **Novarsenobillon**

H₂N NH.CH₂OSONa

OH OH

As ═ As **Arsenophenyl-glycine**

NH.CH₂.COONa NH.CH₂.COONa

As ═ As **Arsenophenyl-glycine amide**

NH.CH₂.CONH₂ NH.CH₂.CONH₂

As $\overset{O}{\underset{ONa}{\diagup}}$ **Sodium arsenite**

CH.OH.COOK
|
CH.OH.COO.SbO **Antimony potassium tartrate**

$\overset{O}{\overset{\|}{HO-Sb-ONa}}$ **Stibenyl**

NH.CO.CH₃

$\overset{O}{\overset{\|}{HO-Sb-ONa}}$ **Stibosan**

Cl

NH.CO.CH₃

Phenol

OH

Aniline

NH₂

Acriflavine

H₂N NH₂

N

CH₃ Cl

therapeutic activity requires no other explanation than that it is solely dependent on the direct action of the drug on the parasite. A single illustration will indicate the reasonableness of this view. A dose of 0·05 mgm. of halarsol in 1 c.c. of water given intraperitoneally to a 20 gm. mouse, the blood of which shows a moderate infection of trypanosomes (about 30,000 per c.mm.) will sterilize the peripheral blood within an hour or two. It is not possible to state what is the maximum concentration of the drug in the circulation of the mouse after an injection of 0·05 mgm. but it is not likely to exceed 1 : 30,000 and probably is considerably less, possibly even a tenth of this, i.e., 1 : 300,000. Nevertheless as repeated *in vitro* experiments have shown that a concentration of 1 : 3,200,000 halarsol is sufficient to kill the great majority of the trypanosomes in a suspension of 30,000 per cmm. within an hour or two, there seems no reason to invoke any other than direct action as the true explanation of the mechanism of the therapeutic effect of halarsol in the living animal.

In view of the inactivity of the pentavalent arsenicals and of the remarkable activity of the corresponding reduction products, the hypothesis that the pentavalent compounds owe their therapeutic activity to the fact that they are in part reduced in the body of the host is not unreasonable. The fact that the trivalent compounds produce peripheral sterilization within an hour or so, whereas the sterilizing effect of the corresponding pentavalent compounds is first manifest after an interval of six or seven hours, affords support for the view.

The reaction of the two resistant strains to the various drugs discloses a number of highly interesting facts. In the first place, it is worthy of remark that the two strains, made resistant by the use of such widely different compounds as atoxyl and acriflavine, should prove themselves exactly similar in their reaction to the 21 compounds which we have examined. This seems to us to be a matter of importance and we shall return to it later in the present paper.

In view of the fact that the trypanocidal action exerted by aniline, phenol and the pentavalent aresenicals on the normal strain is so slight, as to cause one to doubt whether they have any specific trypanocidal action at all, it is not surprising that the resistant

characters of the two resistant strains is but slightly manifest in respect of these compounds. Broadly speaking, the resistant strains proved themselves about twice as resistant to all of these compounds as did the normal strain. When, however, we turn to the highly trypanocidal trivalent compounds, we find a totally different state of affairs. To each of these the resistant strains exhibited a very definite degree of resistance, but strangely enough the degree of resistance to the various compounds differed enormously. In the case of *p*-aminophenylarsenoxide and reduced atoxyl thioglycollate the resistant strains withstood 4 to 8 times, in that of reduced stovarsol thioglycollate about 8 to 16 times, in that of halarsol, halarsol thioglycollate and reduced arsacetin about 32 to 64 times, and in that of reduced tryparsamide thioglycollate no less than about 1,024 times the concentration which destroyed the normal strain. The same pronounced differences were seen in respect of the arsenobenzols, the resistant strains tolerating 32 to 64 times as strong solutions of novarsenobillon as did the normal strain, in the case of arsenophenylglycine only 2 to 4 times as much, but in that of the amide of this compound (arsenophenylglycine amide) no less than about 256 times as much as did the normal strain.

Before passing to consider these very suggestive facts, attention should be drawn to the important observation previously referred to, viz., that the resistant strains proved just as sensitive to the action of sodium arsenite and of tartar-emetic as did the normal strain. This observation has the more weight in that each of the compounds in question is highly trypanocidal, sodium arsenite killing all the strains within twenty-four hours when diluted 3,200,000 times, and tartar-emetic when diluted 6,400,000 times.

This fact, that neither of the resistant strains exhibit the slightest resistance to sodium arsenite, seems capable of only one interpretation, viz., that the expression *arsenic resistance* as applied to atoxyl-resistant and acriflavine-resistant strains is a definite misnomer. These strains are not *arsenic resistant* but are resistant to various organic compounds of arsenic.

This conclusion is amply supported by the facts we have just summarized relating to the behaviour of the atoxyl- and acriflavine-resistant strains to the various trivalent arsenicals and arsenobenzols.

All these compounds contain, within, of course, certain rather narrow limits, approximately the same relative amount of arsenic trioxide, and yet to some of them, viz., *p*-aminophenylarsenoxide, reduced atoxyl thioglycollate and arsenophenylglycine, the resistant strains are only 4 to 8 times as resistant as the normal strains ; whereas, to quote the other extreme, to reduced tryparsamide thioglycollate and arsenophenylglycineamide the resistant strains are no less than 256 to 1,024 times as resistant as the normal strain. These facts, taken in conjunction with the lack of resistance exhibited by the resistant strains to sodium arsenite, can only mean that the resistance is really to the substituted phenyl radical. It is the constitution and position of the side chain which determines the trypanocidal activity of a trivalent arsenical, or of an arsenobenzol compound ; e.g., reduced stovarsol thioglycollate and arsenophenylglycine are hardly more active than sodium arsenite itself, whereas the other trivalent arsenicals and arsenobenzols are at least 30 to 60 times as active. Similarly, it is the constitution and position of the side-chain which determines the degree of resistance exhibited by the resistant strains.

It is impossible from the limited number of compounds we have so far examined—unfortunately, the procedure is exceedingly laborious, involving long stretches of continuous work—to make any generalization on the significance of the constitution and position of the side-chain in the activity of the compound ; it is obvious that before we shall be in a position to do this, many more products must be tested. We might, however, draw attention to one or two outstanding facts.

Firstly, it is interesting that so little resistance is exhibited by the atoxyl-resistant strain to the reduced product of the very drug used to make the strain resistant. We might well have anticipated that the atoxyl-resistant strain would have proved more resistant to reduced atoxyl thioglycollate and to *p*-aminophenylarsenoxide than to any of the other organic arsenicals ; yet the very reverse is the case.

Secondly, the substitution of the –NH$_2$ group in the *para* position (reduced atoxyl thioglycollate) by a glycineamide group in the same position (reduced tryparasamide thioglycollate), although not producing any marked effect on the trypanocidal activity for the

normal strain, so lessens the activity for the resistant strains that the resistant factor of these is raised from about 8 in the case of reduced atoxyl-thioglycollate to about 1,024 in that of reduced tryparsamide thioglycollate. On the other hand, changing the glycine side-chain of arsenophenylglycine into glycineamide as in arsenophenylglycine-amide not only enormously increases the trypanocidal activity for the normal strain, but actually lessens the activity for the resistant strains, so that the resistant factor changes from about 2 to about 256. It is highly interesting to observe that the resistant strains exhibit such an enormous degree of resistance to the reduced product of tryparsamide —a drug of such exceptional value in the treatment of certain types of human trypanosomiasis. This anomaly we are at present unable to explain but it obviously opens up an important field for research.

Probably the information which will be accumulated as the result of study of the trypanocidal action for normal and resistant-strains of a larger number of compounds will throw much new light on the enormously important, and, as yet, very obscure, question of drug resistance. The fact that parasites develop a resistance to certain drugs and that this resistance varies in the case of different drugs, is one of fundamental significance in therapy.

SUMMARY

1. As a preliminary step in an investigation designed with the object of throwing light on the mechanism of their curative action in the infected animal, the trypanocidal action of a number of preparations of arsenic and antimony was examined *in vitro*.

2. The compounds employed were atoxyl, arsacetin, tryparsamide, stovarsol, halarsol, halarsol thioglycollate, *p*-aminophenyl-arsenoxide, reduced atoxyl thioglycollate, reduced arsacetin, reduced tryparsamide thioglycollate, reduced stovarsol thioglycollate, novar-senobillon, arsenophenylglycine, arsenophenylglycineamide, sodium arsenite, stibenyl, stibosan, potassium antimony tartrate, phenol, aniline, and acriflavine.

3. The trypanocidal action *in vitro* of these drugs was examined on a normal strain of *T. rhodesiense* and on atoxyl- and acriflavine-resistant varieties of this parasite.

4. With the normal strain of trypanosomes it was found that the organic pentavalent compounds are but slightly trypanocidal, a solution of 1 : 1600 being required to destroy the parasites within 24 hours. Aniline and phenol were several times more active. The organic trivalent arsenical compounds are extraordinarily trypanocidal, even when diluted several hundred million times they killed the trypanosomes within 24 hours ; this also applies in the case of the arsenobenzols—novarsenobillon and arsenophenylglycineamide—but arsenophenylglycine, although very active, is much less so than the other arsenobenzols, a solution of 1 : 3,200,000 being required to destroy the trypanosomes in 24 hours. Sodium arsenite and tartaremetic likewise displayed considerable action, the corresponding trypanocidal titres being respectively 1 : 3,200,000 and 1 : 6,400,000.

5. The response of each of the two resistant strains to the various compounds, although frequently very different from that of the normal strain, proved in every case to be exactly similar to one another. The resistant strains were but slightly more resistant to the pentavalent arsenicals and to aniline than was the normal strain, withstanding as a rule about twice the concentration of drug as did the latter strain. But to the various trivalent arsenicals and arsenobenzols the resistant strains exhibited extraordinarily different degrees of resistance, e.g., to reduced atoxyl thioglycollate, *p*-aminophenylarsenoxide and arsenophenyglycine, they were only about 4 or 8 times as resistant as was the normal strain, whereas to arsenophenylglycineamide and to reduced tryparsamide thioglycollate they were no less than 256 to 1024 times as resistant as was the normal strain. To sodium arsenite and to tartar-emetic the resistant strains proved to be just as susceptible as did the normal strain.

6. From these facts the following deductions are drawn :—

(1) The trypanocidal activity *in vitro* of the organic trivalent arsenicals, of the arsenobenzols, of sodium arsenite, tartar-emetic and acriflavine is such as to warrant the conclusion that their therapeutic activity in the infected vertebrate requires no other explanation than that it is solely due to the direct action of the drug on the parasite. The action of the organic pentavalent arsenical and antimonial compounds is so slight as to render such an explanation of their therapeutic activity untenable. A reasonable

explanation of their therapeutic value appears to be that they are reduced in the body of the host to the corresponding trivalent compounds.

(ii) The term ' *arsenic resistance* ' as applied to atoxyl-resistant and acriflavine-resistant strains is a definite misnomer. These strains are not arsenic resistant, but resistant to various organic compounds of arsenic. The similarity of the behaviour of the two strains made resistant to atoxyl and acriflavine respectively, the varying degree of resistance which they exhibit to the different organic compounds of arsenic, and the fact that they are just as susceptible to sodium arsenite as is the normal strain, all indicate in the clearest possible manner that the resistance of these strains is not to arsenic, but to the substituted phenyl radical.

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A CASE OF QUININE HAEMOGLOBINURIA

BY

WARRINGTON YORKE

The following notes, concerning a case in which the administration of small doses of quinine was repeatedly followed by haemoglobinuria, appear to be worthy of record.

The patient, a daughter of English parents, was born at Broken Hill, N. Rhodesia, in April, 1927. Six weeks later she was taken to a farm 50 miles from Fort Jameson ; during the journey prophylactic quinine, in the form of daily doses of half a grain of euquinine, was commenced, and was continued after her arrival at her destination. The child remained quite well, except for a single attack of fever lasting three or four days, when she was about a year old, until June, 1929. During the whole of this period, June, 1927, to June, 1929, she had been given the daily half grain dose of quinine, except during the cold months, April to July.

In June, 1929, the child was taken to stay with some friends in Fort Jameson. A few days later she developed an attack of fever and was given quinine, half a grain, daily. She rapidly improved and a week later returned home and remained well until September, 1929, when one morning her mother, who noticed that the child ' was very restless and cross,' gave her a dose of one grain of quinine about 10 a.m. During the afternoon of the same day the child developed an attack of blackwater and was immediately taken to the doctor in Fort Jameson. The following day the urine had cleared ; she was again given one grain of quinine, and this in turn was followed by another attack of haemoglobinuria. The child was then admitted to hospital in Fort Jameson for two weeks ; the haemoglobinuria disappeared in the course of a day or two, and she rapidly recovered. No more quinine was given during this period.

She was then removed from hospital to the house of friends in Fort Jameson and a week later the doctor advised commencing quinine again. She was consequently given a dose of one grain,

and this once more was followed by a mild attack of blackwater. No more quinine was given and a fortnight later she was taken home to the farm and remained well up to December, 1929, when it was decided that her mother should take her to England.

On the way to Beira they stayed at Blantyre for a few days, awaiting a train, and the mother took the opportunity of consulting the Medical Officer; he advised that, although the child appeared well, she should be given small doses of quinine to prevent the possibility of an attack of malaria on the voyage home. The child was consequently given half a grain of quinine, and three hours later passed blackwater; she was admitted to hospital and all the symptoms subsided in the course of a day or two.

At the beginning of January, 1930, whilst in the Red Sea, the child appeared to be slightly indisposed and the ship's surgeon advised quinine. She was given a dose of half a grain, and again had another mild attack of blackwater.

No further quinine was given and the child rapidly improved and remained well until she was brought to see me at the end of July, 1930, with a view to obtaining an opinion whether she was fit to return to Central Africa. She was then in excellent health, well-nourished, not anaemic, there was no enlargement of the spleen, the urine was normal, and examination of the blood failed to reveal anything suggestive of malaria. I expressed the opinion that, in view of the fact that the child had suffered from so many attacks of haemoglobinuria as the result of small doses of quinine, it would be unwise for her to return to a malarious region. This advice greatly distressed the mother, who was exceedingly anxious to take the child back to Rhodesia with her. In view of the mother's distress, I suggested that possibly, as the child had been perfectly well during the last seven months, she might no longer be hyper-sensitive to quinine, and that it might be worth while ascertaining whether she could now take quinine without ill effects. It was consequently decided to try the experiment.

She was given half a grain of quinine daily on August 1st, 2nd and 3rd, without any ill effect. On August 4th, the dose was increased to one grain, and a similar dose was given on the morning of August 5th. During the evening of this day she became feverish and very cross, and early the next morning passed blackwater and

became definitely icteric ; the urine gradually cleared during the day and the child rapidly recovered.

The following points in this interesting case seem worthy of note :—

During the first two years of life the child was apparently not hyper-sensitive to quinine, and indeed took the drug regularly without ill effect. It was not until after the second definite attack of malaria that she became hyper-sensitive to the drug. From this period onwards, the administration of the same dose, which she had previously taken with impunity, invariably produced an attack of haemoglobinuria. Possibly during her seven months' sojourn in England, without malaria and without quinine, the hyper-sensitiveness to quinine had to some extent decreased. This is suggested by the fact that it was only after the administration of quinine for five days (half a grain on each of the first three days, and one grain on each of the last two) that she developed blackwater, whereas on each of the five previous occasions a single dose varying from half to one grain had sufficed to provoke an attack of haemoglobinuria.

ANNALS

OF

TROPICAL MEDICINE AND

PARASITOLOGY

THE INCORPORATED
LIVERPOOL SCHOOL OF TROPICAL MEDICINE

FOUNDED BY SIR ALFRED LEWIS JONES, K.C.M.G

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The following courses of instruction are given by the Liverpool School of Tropical Medicine each year :—

(1) Two courses for the Diploma in Tropical Medicine, commencing on the 5th January and the 1st October, 1931. The D.T.M. examinations are held in December and March.

(2) Two courses for the Diploma in Tropical Hygiene, commencing on the 12th January, and the 22nd April, 1931. The D.T.H. examinations are held in March and July.

(3) Two courses in Veterinary Parasitology, commencing on 5th January and the 1st October, 1931.

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This Diploma shall be awarded only to candidates who possess a qualification to practise Medicine recognised for this purpose by the University, and who present satisfactory certificates of having attended approved courses of study, and pass the prescribed examination.

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This Diploma can only be taken by those who have already obtained the D.T.M. of the University of Liverpool.

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For prospectus and further information, application should be made to the Hon. Dean, School of Tropical Medicine, University of Liverpool.

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1909	Murphy, John Cullinan
1909	Samuel, Mysore Gnananandaraju
1909	Shroff, Kawasjee Byramjee
1909	Thornely, Michael Harris
1909	Turkhud, Violet Ackroyd
1909	Webb, William Spinks
1909	Yen, Fu-Chun
1910	Brabazon, Edward
1910	Castellino, Louis
1910	Caulcrick, James Akilade
1910	Dowden, Richard
1910	Haigh, William Edwin
1910	Hamilton, Henry Fleming
1910	Hefferman, William St. Michael
1910	Hipwell, Abraham
1910	Homer, Jonathan
1910	Houston, William Mitchell
1910	James, William Robert Wallace
1910	Johnstone, David Patrick
1910	Korke, Vishnu Tatyaji
1910	Macdonald, Angus Graham
1910	Macfie, John Wm. Scott
1910	Manuk, Mack Walter
1910	Murison, Cecil Charles
1910	Nanavati, Kishavlal Balabha
1910	Nauss, Ralph Welty
1910	Oakley, Philip Douglas
1910	Pratt, Ishmael Charles
1910	Sabastian, Thiruchelvam
1910	Shaw, Hugh Thomas
1910	Sieger, Edward Louis
1910	Sousa, Pascal John de
1910	Souza, Antonio Bernardo de
1910	Waterhouse, John Howard
1910	White, Maurice Forbes
1911	Blacklock, Donald Breadalbane
1911	Brown, Frederick Forrest
1911	Chand, Diwan Jai
1911	Holmes, John Morgan
1911	Ievers, Charles Langley
1911	Iles, Charles Cochrane
1911	Ingram, Alexander
1911	Kirkwood, Thomas
1911	Knowles, Benjamin
1911	Liddle, George Marcus Berkeley
1911	Lomas, Emanuel Kenworthy
1911	Mackarell, William Wright
1911	MacKnight, Dundas Simpson
1911	Mascarenhas, Joseph Victor
1911	Murray, Ronald Roderick
1911	Oluwole, Akidiya Ladapo
1911	Rao, Koka Ahobala
1911	Sinton, John Alexander
1911	Tarapurvalla, Byramji Shavakshah
1911	Taylor, John Archibald
1911	Woods, William Medlicott
1912	Aeria, Joseph Reginald
1912	Anderson, Edmund Litchfield
1912	Borle, James
1912	Bowie, John Tait
1912	Brassey, Laurence Percival

1912	Christie, David
1912	Dillon, Henry de Courcy
1912	·Dunn, Lillie Eleanor
1912	Hardwicke, Charles
1912	Jagose, Jamshed Rustomji
1912	Kochhar, Mela Ram
1912	McGusty, Victor William Tighe
1912	Milne, Arthur James
1912	Mitra, Manmatha Nath
1912	Myles, Charles Duncan
1912	Pelly, Huntly Nevins
1912	Prasad, Bindeshwari
1912	Prentice, George
1912	Ross, Frank
1912	Russell, Alexander James Hutchison
1912	Ruthven, Morton Wood
1912	Sandilands, John
1912	Seddon, Harold
1912	Smalley, James
1912	Strickland, Percy Charles Hutchison
1912	Watson, William Russel
1913	Austin, Charles Miller
1913	Banker, Shiavux Sorabji
1913	Becker, Johann Gerhardus
1913	Carrasco, Milton
1913	Clark, James McKillican
1913	Forsyth, Charles
1913	Grahame, Malcolm Claude Russell
1913	Grieve, Kelburne King
1913	Hargreaves, Alfred Ridley
1913	Hepper, Evelyn Charles
1913	Hiranand, Pandit
1913	Jackson, Oswald Egbert
1913	Khaw, Ignatius Oo Kek
1913	MacKelvie, Maxwell
1913	MacKinnon, John MacPhail
1913	Macmillan, Robert James Alan
1913	Mouat-Biggs, Charles Edward Forbes
1913	Noronha, John Carmel
1913	O'Connor, Edward
1913	Olubomi-Beckley, Emanuel
1913	Pestonji, Ardeshir Behramshah
1913	Puttanna, Dodballapur Sivappa
1913	Reford, John Hope
1913	Smith, Edward Arthur
1913	Stewart, Samuel Dudley
1913	Walker, Frederick Dearden
1913	Wilbe, Ernest Edward
1913	Wilson, Hubert Francis
1913	Yin, Ulg Ba
1913	Young, William Alexander
1914	Arculli, Hassan el
1914	Chohan, Noormahomed Kasembha
1914	Connell, Harry Bertram
1914	Gerrard, Herbert Shaw
1914	Gimi, Hirji Dorabji
1914	Gwynne, Joseph Robert
1914	Hodkinson, Samuel Paterson
1914	Jackson, Arthur Ivan
1914	Kaushash, Ram Chander
1914	Kelsall, Charles
1914	Luanco y Cuenca, Maximino
1914	Misbah, Abdul-Ghani Naguib

Date	Name
1914	Naidu, Bangalore Pasupulati Balakrishna
1914	Rowe, John Joseph Stephen
1914	Roy, Raghu Nath
1914	Shiveshwarkar, Ramchandra Vishnu
1914	Sur, Sachindra Nath
1914	Talati, Dadabhai Cursedji
1914	Wilkinson, Arthur Geden
1914	Wright, Ernest Jenner
1915	Lobo, John Francis
1915	Madhok, Gopal Dass
1915	Pearson, George Howorth
1915	Swami, Karumuri Virabhadra
1915	Wood, John
1916	Barseghian, Mesroob
1916	Chaliha, Lakshmi Prasad
1916	Lim, Albert Liat Juay
1916	Lim, Harold Liat Hin
1916	Metzger, George Nathaniel
1916	Söderström, Erik Daniel
1916	Wheeler, Louis
1917	Chapman, Herbert Owen
1917	Krishnamoorthy, Yedatore Venkoba
1917	Lipkin, Isaac Jacob
1918	Watts, Rattan Claud
1919	Bowle-Evans, Charles Harford
1919	Burnie, Robert McColl
1919	Celestin, Louis Abel
1919	Cummings, Eustace Henry Taylor
1919	Darling, Georgina Renington
1919	Drake, Joan Margaret Fraser
1919	Fraser, William James
1919	Gordon, Rupert Montgomery
1919	Krige, Christian Frederick
1919	Maplestone, Philip Alan
1919	Oluwole, Isaac Ladipo
1919	Rustomjee, Khusshuyee Jamesidjee
1919	Sawers, William Campbell
1919	Thompson, Mary Georgina
1919	Turner, Gladys Maude
1919	Young, Charles James
1920	Adler, Saul
1920	Anderson, William Jenkins Webb
1920	Campbell, George
1920	Cobb, Charles Eric
1920	Cobb, Enid Margaret Mary
1920	Connolly, Evelyn Mary
1920	Fernandez, Daniel David
1920	Lim, Chong Eang
1920	McHutcheson, George Browne
1920	van der Merwe, Frederick
1920	O'Farrell, Patrick Theodore Joseph
1920	Renner, Edowo Awunor
1920	Vaughan, James Churchwill
1920	Waller, Harold William Leslie
1921	Allen, George Phillip Farmer
1921	Corfield, Charles Russell
1921	Hamid, Abdul
1921	Longhurst, Bell Wilmott
1921	Macvae, George Anthony
1921	Madan, Hans Raj
1921	Mulligan, William Percival

Date	Name
1921	Nixon, Robert
1921	Richmond, Arthur Stanley
1921	Shri Kent, Shamsher Singh
1921	Skinner, James Macgregor
1921	Stewart, Robert Bell
1921	Thomson, Marion
1922	Bhatia, Jagat Ram
1922	Cohen, Morris Joshua
1922	Crawford, Andrew Clemmey
1922	Gilmore, Edward Raymond
1922	Gracias, Cajetan Manuel
1922	Jennings, Arthur Richard
1922	Lethem, William Ashley
1922	Paul, Sachchidananda Hoshen
1922	Pinder, John
1922	Rieley, Stanley Desmond
1922	Rutherford, Gladys
1922	Stewart, Quintin
1923	Abelman, B.
1923	Basu, Dhirendranath
1923	Cruickshank, John Cecil
1923	Doherty, Winifred Irene
1923	Edghill, Winifred M.
1923	Elsohn, John
1923	Fraser, N. D.
1923	Lee, R.
1923	Pierce, E. R.
1923	Raja, Rojaporum
1923	Reid, C. B. B.
1923	Richmond, A. E.
1923	Steven, J. B.
1923	White, Charles Francis
1924	Bilimoria, H. S.
1924	Carson, J. C.
1924	Chopra, B. L.
1924	Davis, B. L.
1924	Hardy, M. J.
1924	Jennings, C. B.
1924	Johnstone, F. J. C.
1924	Keirans, J. J.
1924	Lee, S. W. T.
1924	Macdonald, G.
1924	Maclean, G.
1924	Mathur, W. C.
1924	Mitchell, J. M.
1924	Owen, D. Uvedale
1924	Palmer-Jones, Beryl
1924	Sankeralli, E. J.
1924	Singh, H.
1924	Theron, Elizabeth M.
1925	Adams, Alfred Robert Davies
1925	Ashton, Frank Richard
1925	Ashworth, Esther
1925	Bamford, Charles Walker
1925	Beinashowitz, Jack
1925	Black, John
1925	Clark, George
1925	Coghlan, Bernard A.
1925	Collier, Ivy
1925	Crawford, E. J.
1925	Cumming, Patrick Grant

1925	Ellam, Mary Muriel
1925	Fisher, Morris
1925	Green, Frederick Norman
1925	Grutu, M. S.
1925	Hawe, Albert J.
1925	Jafri, Z. H.
1925	Johnstone, Elvy I.
1925	Kerr, James R.
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1925	Mackay, E. K.
1925	Makkawi, M.
1925	Maldonado, Leopoldo Garcia
1925	Mar, Severo Francisco
1925	Mozoomdar, B. P.
1925	Shah, Khwaja Samad
1925	Skan, Douglas A.
1925	Stone, Ernest R.
1925	Terrell, C. G.
1925	Tooth, Frederick
1925	de Waal, Jacobus Johannes
1926	Aitken, W. J.
1926	Ashworth, A.
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1926	Doherty, H. A. A.
1926	Don, E. G.
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1926	Fletcher, Beatrice N.
1926	Fowler, H. P.
1926	Fowler, Isabella J.
1926	Hamilton, J.
1926	Hodgkinson, Katharine M.
1926	Jackson, R.
1926	Kamakaka, K. H.
1926	Kennedy, J. H.
1926	Khatri, L. D.
1926	Lennox, D.
1926	Lewis, A. J.
1926	McConn, C. F.
1926	Mackay, A. G.
1926	McLean, N.
1926	MacSweeney, M.
1926	Malhautra, K. L.
1926	Malik, S. B.
1926	Manuwa, S. L. A.
1926	Merchant, M. E.
1926	Mitchell, W. H.
1926	Molony, E. F.
1926	Nashikkar, S. G.
1926	Oppenheimer, F.
1926	Ormiston, W. S.
1926	Paterson, F. S.
1926	Patterson, F. L.
1926	Pouri, V.
1926	Quigley, L. D.
1926	Robertson, A.

1926	Rodrigues, N.
1926	Sachdev, A. S.
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1926	Singh, J.
1926	Talib, S. A.
1926	Tan, C. L.
1926	Taylor, Catherine F.
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1926	Turner, J. G. S.
1926	Vardya, B. K.
1926	Varma, T. N.
1926	Voigt, C.
1926	Wasti, S. N.
1927	Allen, C. P.
1927	Bahl, M. L.
1927	Barrowman, B.
1927	Bawa, H. S.
1927	Bilimoria, J. D.
1927	Burns, W. M.
1927	Daly, E. J.
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1927	Hay, R.
1927	Hodivala, N. M.
1927	Hughes, Emma
1927	Hyslop, Kathleen M.
1927	Ingram-Johnson, R. E.
1927	Kapadia, J. S.
1927	Khan, F. A.
1927	Khan, M. M.
1927	Labuschagne, P. N. 11.
1927	Laird, W. J.
1927	Lewin, B. F.
1927	Macdonald, J.
1927	McElroy, R. S.
1927	Maclay, W. S.
1927	Maguire, H. G.
1927	Mahaffy, A. F.
1927	Malhotra, A. H.
1927	Malhotra, A. L.
1927	Manghirmalani, B. S.
1927	Meek, A. I.
1927	Mehra, J. N.
1927	Mehta, H. C.
1927	Menon, M. V.
1927	Miller, H. V. R.
1927	Mokand, S. N.
1927	Murgatroyd, F.
1927	Murray, A. J.
1927	Murray, Pauline V.
1927	Nevin, H. M.
1927	Nirula, P. N.
1927	Olusoga, N. T.
1927	Parakh, D. B.
1927	Peters, D. O.
1927	Peters, M. R.
1927	Pottinger, J. H.
1927	Rao, R. S.
1927	Rodriguez, G. V. S.
1927	Shah, S. R. A.

The following have obtained the Diploma in Tropical Hygiene of the University of Liverpool :—

Diploma in Tropical Hygiene

SOME PARASITIC NEMATODES OF FROGS AND TOADS ·

BY

J. N. KARVE, M.Sc.

PROFESSOR OF BIOLOGY, SIR PARASHURAMBHAU COLLEGE, POONA, INDIA

The present paper describes three species of Nematodes, two of which were parasitic in the frog (*Rana tigrina*) and were collected by the writer at Nagpur.   The third was obtained by Professor G. D. Bhalerao, of Science College, Nagpur, from the toad (*Bufo melanostictus*), at Rangoon (Burma), and very kindly handed over by him to the writer for determination.

Family HETERAKIDAE.
Sub-family HETERAKINAE.
Meteterakis govindi, n.g., n.sp.

Worms belonging to this species are small in size.   The body is bent ventrally to a marked degree in both sexes.   Lateral alae are present, but they could not be observed very well in the male. In the female they are feebly developed and beginning at 0·3075 mm. from the anterior extremity continue to the tip of the tail.   The transverse striations could not be observed.

The mouth is surrounded by three lips, one dorsal and two sub-ventral.   Each lip carries a single papilla.   The diameter of the head, at the base of the lips, is 0·62 to 0·3512 mm.   A small oral cavity is present.   The oesophagus is divided into three parts. The first of these, the pharynx, measures about 0·0432 mm. in the male and 0·0448 to 0·0544 mm. in the female.   The breadth of the pharynx is about 0·025 mm. in the male and 0·0288 mm. in the female. The pharynx is distinctly separated from the following portion of the oesophagus by a partition.   The second portion of the oesophagus is a strongly muscular simple tube, which passes posteriorly into the

third portion of the oesophagus, the bulb. The lumen of this tube is triradiate and is lined throughout its length by thick refractive cuticle. The oesophageal bulb is highly muscular and contains the valvular apparatus. It measures 0·19 to 0·3 mm. by 0·143 to 0·193 mm. The lumen of the bulb is also lined by thick refractive cuticle. No intestinal valves could be detected at the junction of the oesophagus and the intestine. The entire oesophagus measures 0·887 to 1·0 mm. in the male and 0·931 to 1·043 mm. in the female.

No cervical papillae were observed. The nerve-ring cannot easily be located and appears to encircle the oesophagus at about 0·2775 to 0·3 mm. from the anterior end. The excretory pore is very large and is situated at a distance of 0·462 mm. in the male and 0·512 to 0·54 mm. in the female, from the same end. It communicates with a big sac into which two canals open.

The male is 5·0 to 5·4 mm. in length, and has a maximum thickness of 0·231 to 0·287 mm. measured dorsoventrally. The posterior end possesses well-developed alae which form the bursa. There is a preanal sucker with distinctly chitinized rims. It measures 0·0415 to 0·044 mm. by 0·024 to 0·0298 mm., and is situated at a distance of 0·0687 mm. anterior to the cloaca. The caudal papillae are numerous. Some of them are very minute and extremely difficult to detect. Seventeen pairs could definitely be observed. They can be divided into presuctorial, parasuctorial and postsuctorial groups. The presuctorial group consists of four pairs of papillae, all of which are ventral, but in certain specimens two out of the four pairs are slightly lateral. There are three pairs of papillae in the parasuctorial group, all of which are ventrolateral. The two anterior pairs of this group are very stout the third being much slenderer. The postsuctorial group of papillae falls into two sub-groups, one preanal and the other postanal. The preanal group is made up of three pairs two of which are ventral and one lateral and very stout. The stout lateral pair may be situated by the side of the cloacal opening or a little anterior to it. One of the two ventrally situated pairs of this group is very small. The postanal group consists of seven pairs of small papillae. Of the posterior three pairs two are subventral. The remaining five pairs are all ventrally situated. The area surrounding the cloacal aperture appears to be covered with minute papillae besides those mentioned.

The equal and similar spicules measure about 0·27 mm. In the latter half of their length they are very delicate. The cloacal opening situated at 0·168 to 0·2 mm. from the posterior end, is very large and has well-developed lips as a result of which the cloaca seems to protrude out of the body when viewed laterally.

The female measures 4·6 to 6·0 mm., having a dorsoventral thickness of 0·250 to 0·3 mm. The vulva lies a little anterior to the middle of the body. The body of the female gradually tapers down towards both the extremities from this region. In three specimens measuring 4·6 mm., 5·6 mm., and 6·0 mm. the vulva was situated at 2·2 mm., 2·5 mm., and 2·8 mm. respectively from the anterior end. The opening of the vulva is protected by a strongly developed flap which is directed posteriorly. The vagina is long, muscular, and well developed. It runs posteriorly and parallel to the body wall. At a point about 1·3 mm. from the vulva it bifurcates into two uterine branches, which appear to be parallel. The branches of the uterus are packed with eggs measuring 0·075 by 0·0431 mm. The shell measures 0·0048 mm. In a specimen measuring 4·6 mm. the most anterior loop of the ovary is situated at a distance of 0·3375 mm. from the posterior end of the oesophagus and the most posterior loop of the uterus is situated at 0·675 mm. from the caudal extremity. It is very difficult to follow the complete course of the genitalia as the uterine branches are densely packed with eggs and appear to fill nearly the whole of the body. The tail is 0·262 to 0·343 mm. long and gradually tapers down to a point. The caudal papillae are situated at a distance of 0·09 mm. from the posterior end.

Affinities. This worm is closely related to *Heterakis* in the possession of caudal alae, three lips, an oesophagus terminating posteriorly in a bulb, a preanal sucker with chitinoid border and a well-developed bursa ; but differs from it in possessing seventeen pairs of caudal papillae, feebly developed lateral alae, comparatively long pharynx, flapped vulva and long, muscular vagina.

Generic diagnosis. Heterakinae ; cuticle with feebly developed lateral alae. Mouth with three lips, each carrying a single papilla. Male : caudal alae well developed ; seventeen pairs of caudal papillae ; four pairs presuctorial, three parasuctorial, and ten post-suctorial ; two parasuctorial pairs and one postsuctorial (adanal or a little anterior to the anal aperture)—very stout. Spicules equal,

similar and delicate in the latter half of their length.　Female :
Vulva a little anterior to the middle of the body, uterine branches
parallel, oviparous ; eggs with unsegmented contents.　Parasites of
toads.

Type species :—*Meteterakis govindi*.
Host :—*Bufo melanostictus*.
Habitat :—Rectum.
Locality :—Rangoon (Burma).
This species is named after Professor Bhalerao.
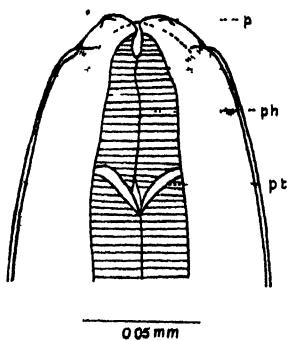
FIG. 1.　Anterior end of a female worm
seen from the ventral side.　*p.*—Papilla situated
on the lip ;　*ph.*—Pharynx ;　*pt.*—Partition
separating the pharynx from the middle
portion of the oesophagus.
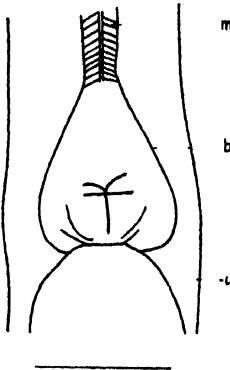
FIG. 2.　Last portion of the oesophagus—
the bulb.　Ventral view.　*b.*—Oesophageal
bulb ,　*i*—Intestine ,　*m*—Middle portion of
the oesophagus.
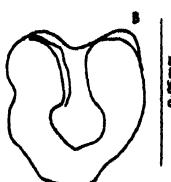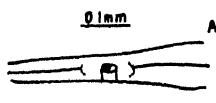
FIG. 3.　*A.*—Excretory pore, lateral view ;　*B.*—Excretory pore, magnified, ventral view.
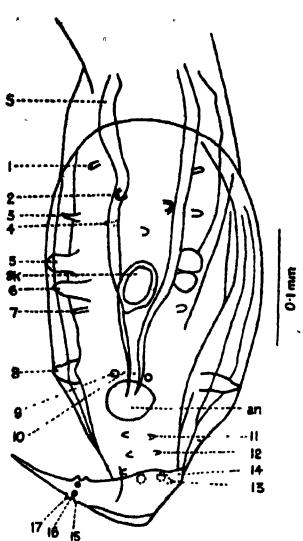
FIG. 4. Posterior end of the male worm showing the caudal papillae, the sucker, the spicules, etc., ventral view. *an.*—Anus; *s.*—Spicule; *sk.*—Sucker; 1-4.—Presuctorial papillae (4 pairs); 5-7.—Parasuctorial papillae (3 pairs); 8-17.—Postsuctorial papillae (10 pairs).
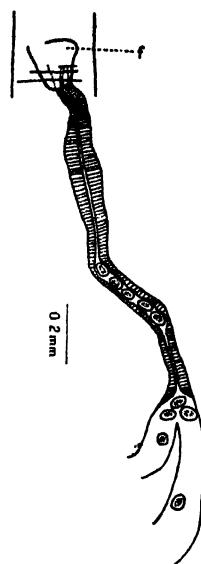
FIG. 5. Female genitalia from a dissected worm. Ventral view. *f.*—Protective flap.
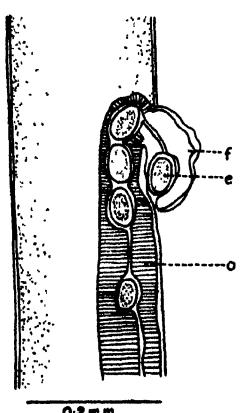
FIG. 6. Region of the vulva. Lateral view. *e.*—Egg; *f.*—Protective flap; *o.*—Vagina.
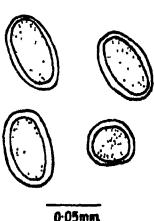
FIG. 8. Eggs.
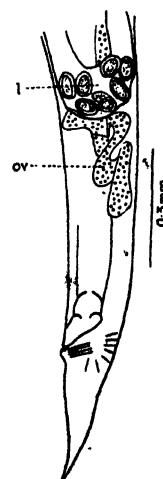
FIG. 7. Lateral view of the posterior end of female. *l.*—Posterior loop of the uterus; *ov.*—Ovary.

Family CAMALLANIDAE.

Camallanus baylisi, n.sp.

The worms, in living condition, had a reddish colour, which was lost after fixing them in 70 per cent. alcohol. The total length is 8·0 to 9·5 mm. in the male, and 14·0 to 20·0 mm. in the female. The male has a maximum thickness of 0·2625 to 0·27 mm., the female 0·4125 mm. The cuticular striations are fine. The dorso-ventral diameter of the head, measured at the anterior angles, is 0·0832 to 0·0975 mm. in the male, and 0·12 to 0·165 mm. in the female. The chitinous buccal valves, broad anteriorly and narrow posteriorly, are distinctly broader than long, their length, excluding the posterior ring, being 0·072 to 0·088 mm. in the male, and 0·1275 to 0·15 mm. in the female. Their width is 0·096 to 0·1168 mm. in the male and 0·1425 to 0·1875 mm. in the female. The number of longitudinal ridges on each valve varies between nine and eleven, the latter number being almost always found in fully developed specimens. The posterior extremity of these ridges (especially of the middle six) is somewhat thick. The ridges at the sides are short and between two ridges is situated a small chitinized tooth-like projection. The posterior ring of the buccal apparatus has a diameter of 0·0656 to 0·0704 mm. in the male, and about 0·08 mm. in the female. In a lateral view the ring appears somewhat curved in the posterior direction. The dorsal and ventral tridents are well developed and the middle prong measures 0·0272 to 0·04 mm. in the male and 0·032 to 0·004 mm. in the female. The tridents show considerable variations in size and shape, some of which are shown in fig. 10. The head bears three papillae on each side near the extremity. The oesophagus shows the usual division into an anterior-muscular and a posterior-glandular portion. The former is distinctly club-shaped, and measures in length 0·48 to 0·5157 mm. in the male and 0·6 to 0·65 mm. in the female. The posterior portion is 0·4350 to 0·54 mm long in the male, and 0·4275 to 0·5175 mm. in the female. The small cervical papillae are situated about 0·1920 mm. from the cephalic end. The nerve-ring encircles the anterior portion of the oesophagus at a distance of 0·1392 to 0·1950 mm. in the male and 0·2325 to 0·2475 mm. in the female from the anterior end. The small excretory pore is situated at 0·31 to 0·3675 mm. from the same end. The intestine is somewhat narrower than the oesophagus.

In the male the bifid (ending in two spines) tail measures 0·16 to 0·18 mm. The caudal end possesses alae, and the alar region of the body is somewhat thick. The alae, broad at the beginning, gradually taper down posteriorly, and at a distance of about 0·2 mm.—in the region of the sixth and the seventh preanal ribs—from the caudal tip they widen out only to taper down again to the extremity. The alae measure 0·7125 to 0·7875 mm. in length. Their width is 0·18 to 0·1875 mm. at the level of the seventh preanal rib and 0·2850 to 0·2925 mm. at the level of the second preanal rib. The ventral region of the bursa is well supplied with muscles the action of which seems to be to produce a sucker-like depression when required.

There are seven pairs of preanal rib-like papillae projecting into the alae. In addition to these there are six pairs of postanal papillae and two pairs of adanal ones. Numbering from the caudal end the first postanal pair is situated at a distance of 0·0225 mm. (male measuring 8·5 mm.) and the second pair at 0·075 mm. from that end. Just anterior to this is situated a pair of small, hair-like, long papillae, with a thick base. The next three pairs form a group and are situated at a distance of 0·1275 mm. from the posterior extremity. The two pairs of adanal papillae appear to be inserted on the lips of the cloaca. The seventh preanal rib is just anterior to the cloacal opening and is 0·1875 mm. distant from the caudal tip. The distance between the sixth and the seventh rib is 0·03 mm. and that between the fourth and the fifth pair is considerable and measures 0·1575 mm. The remaining pairs of the ribs are about equally spaced from each other. The two spicules are unequal and dissimilar. The right is stout and measures 0·4650 mm. in length. It has a prong at the tip which varies in shape. In almost all the specimens the distal portion of this spicule is alate. The left is delicate and very slender and measures 0·149 to 0·225 mm. in length.

The tail of the female measures 0·1275 to 0·1350 mm. and is finger-shaped. At its tip it has three minute spines which are larger in immature specimens. No caudal papillae could be detected. The vulva is in front of the middle of the body, at 5·35 to 6·90 mm. from the anterior extremity. It has prominent, equally developed lips. The vagina is muscular and narrow. It runs back posteriorly, parallel to the body-wall and appears to bifurcate into the opposed

uterine branches at a distance of about 1·5 mm. from the vulva. The posterior branch of the uterus does not end in an ovary as usual and terminates at a distance of 2·80 mm. from the caudal end. The worm is viviparous.

Specific diagnosis. Buccal valves distinctly broader than long, possessing nine to eleven ridges each; the posterior ring of the buccal apparatus slightly curved backwards; tridents vary in shape; caudal alae not very well developed; fifteen pairs of caudal papillae, seven preanal, two adanal, and six postanal. Three pairs of papillae, immediately posterior to the cloacal opening are close together and form a group; a pair of filiform papillae present posterior to this group.

Camallanus kachugae Baylis and Daubney, 1922 is the only other Indian species so far described. The species here described is named after Dr. H. A. Baylis of the British Museum.

Host :—*Rana tigrina.*
Habitat :—Small intestine.
Locality :—Nagpur.

Camallanides prashadi Baylis and Daubney, 1922.

A single male worm, belonging to this species, was obtained from the small intestine of *Rana tigrina.* It is noteworthy that out of about 100 frogs examined for parasites only one yielded a male specimen of this species.

Measurements of this worm are as follows :—

Total length	7.6 mm.
Maximum breadth	0.2175 mm.
Dorsoventral diameter of the head measured at the anterior corners	0.075 mm.
Buccal valves, length	0.0675 mm.
„ „ width	0.0825 mm.
Rods, right	0.05 mm.
„ left	0.04 mm.
Diameter of the Ring	0.0304 mm.
Muscular oesophagus	0.2625 mm.
Posterior oesophagus	0.3750 mm.
Nerve-ring	0.1350 mm. from the anterior end.
Excretory pore	0.2475 mm. from the anterior end.
Cervical papillae	0.2775 mm. from the anterior end.
Tail	0.08 mm. long.
Right spicule	0.24 mm.
Left spicule	0.14 mm.
Accessory piece	0.021 mm.

The caudal end is curved ventrally and is provided with well-developed muscles and alae. There are seven pairs of preanal ribs supporting the alae, and two pairs of ventrally curved adanal papillae. The five pairs of postanal papillae are all lateral. Out of these the three nearer to the cloaca are close together and form a group. The remaining two· are isolated. The spicules are unequal and dissimilar. The right spicule is stout, and in the latter half of its length possesses alae. It forms a hook at its distal end. The left spicule does not possess alae, is slender and delicate and tapers down to a fine point.

Baylis (1929) has published a note on the hosts of this species. He gives a list of hosts which are all reptiles. *Rana tigrina* seems to be a new host for this species.

Host :—*Rana tigrina*.

Locality :—Nagpur, India.
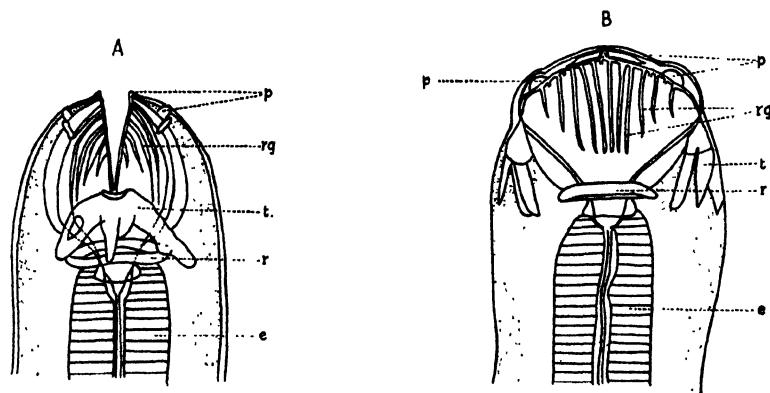
FIG. 9. (*A*).—Dorsal view of the anterior end of a female worm. (*B*).—Lateral view of the same. *p.*—Papillae; *r.*—Ring; *t.*—Trident; *rg.*—Ridges; *e.*—Oesophagus.
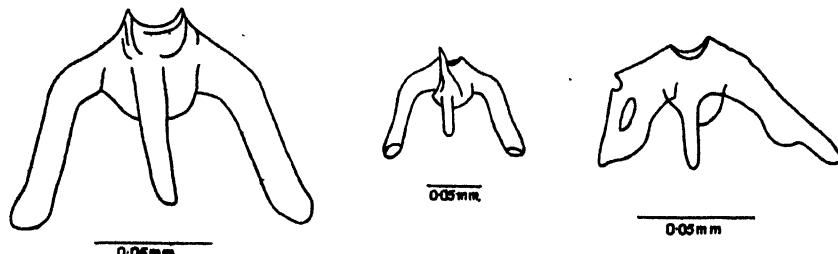
FIG. 10. Tridents showing variations. Ventral view.

0·05 mm.

FIG. 14. The tail region of the male highly magnified to show the first pair of the postanal papillae and the two spines in which the tail ends. Ventral view.

0·3 mm.

0·3 m.m

FIG. 15. Spicules drawn from the ventral side.

FIG. 11. Ventral view of the posterior extremity of a male worm, showing the bursa and the well-developed musculature. b.—Tail ending in two spines; m.—Muscles.
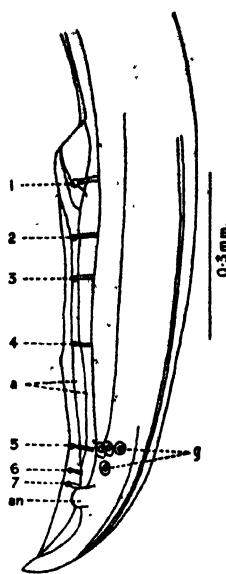
FIG. 12. Lateral view of the posterior end of the male showing the alae, preanal ribs, etc. a.—Alae; an.—Anus; g.—Rectal glands; 1-7.—Preanal ribs.

FIG. 17. The posterior end of an immature female showing the three large spines.

FIG. 13. Ventral view of the posterior region of the male, more magnified, showing the adanal and the postanal papillae. an.—Anus; p', p".—The two adanal papillae; 1-6.—Postanal papillae (Papilla 3 is the hair-like papilla).

FIG. 16. Some variations observed in the distal end of the right spicule.

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POSSIBILITY OF MECHANICAL TRANSMISSION BY INSECTS IN EXPERIMENTAL YELLOW FEVER*

BY

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It has been demonstrated that certain disease-producing organisms are mechanically carried from host to host by blood-sucking insects, most of which are, by the nature of their attack, pre-disposed to intermittent feeding. Mechanical transfer has been particularly clearly shown to occur in sleeping sickness and other trypanosome diseases, and in anthrax. In experiments with these diseases flies have been allowed to bite an infected host and have been interrupted in their feeding and immediately transferred to a normal host, on which they have been permitted to complete their meal. Although mosquitos are known to be the sole vectors of four important human diseases, apparently they have never been shown to act as mechanical disseminators of the causative organisms of these diseases.†

During the investigations of the Yellow Fever Commission in West Africa, it was considered of importance to ascertain whether or not yellow fever virus could be transmitted on the contaminated mouth-parts of mosquitos. Experiments were therefore undertaken using considerably more insects than were likely to be involved epidemiologically in the spread of this disease from any one case. After these investigations were started, there came to hand an interesting paper by Mayne (1928), in which he reported negative results in attempts to transfer tertian malaria mechanically by three to forty specimens of *Anopheles quadrimaculatus* in four trials, and by two to fifteen specimens of *Aedes thibaulti* in eight experiments.

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† Two references have recently been noted, however, in which mosquitos have been considered to act in a mechanical manner in experimental transfer of surra to horses and of fowl-pox. These are respectively: (1) Nieschulz 1928. Zoological contributions to the surra problems. XXII Transmission experiments with *Anopheles fuliginosus*, Gil. [trans. title] *Centbl. Bakt.*, etc. 1 abt. Orig. 109 (5s. 6d.), 327-330. (Abstr. in Exper. Sta. Record, 60, 850.) (2) Kligler, Muchenfuss, and Rivers, 1929. Transmission of fowl-pox by mosquitos. *Jl. Exper. Med.*, 40, 649-660.

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The insects used in the experiments reported below were principally *Aedes aegypti*, but supplemental information is given on *Cimex lectularius* and *Pedicinus* sp. The Asibi strain of yellow fever was employed in all experiments, with the exception that in one with a suspension of monkey lice the A.S. strain was used. In the interrupted feeding of mosquitos and bed-bugs the intervals between successive bites were made as short as possible. The method of immobilizing experimental monkeys on a board, as developed by Stokes, Bauer, and Hudson (1928) was utilized, as it permitted the rapid completion of blood-meals. All source animals died, and the findings at necropsy were typical of yellow fever.[*]

AEDES AEGYPTI Linn.

Three experiments were undertaken with laboratory-reared stock of *Aedes aegypti* Linn. In the first, an infective monkey, *M. rhesus* A, was applied to the outside of the screen cage of a lot of normal mosquitos. When these insects were observed by transmitted light to be definitely obtaining blood, they were drawn into a large pipette and rapidly transferred to another cage *in* which a normal animal, *M. rhesus* B, had been placed. Thirty-nine female mosquitos of Lot 78 were thus transferred during the process of feeding, without apparently seriously inhibiting their desire for further engorgement. Test rhesus B remained normal, and died when again bitten by ten of these insects after the lapse of seventeen days. This confirmed the infectivity of the donor animal as well as the susceptibility and exposure of the test monkey. '

The technique was varied in the second and third experiments in an attempt to obtain greater exposure of the test animals. The source-of-virus monkeys, *M rhesus* (C and G), were placed inside the cages with 139 and 193 normal mosquitos respectively (Lots 138 and 160). The insects had been previously moisture-starved to stimulate rapid feeding. As mosquitos were observed to be partially fed they were blown off the animal with a glass tube inserted through the sleeves of the cages, and normal monkeys D and H were substituted for the infective monkeys. The infective animals

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[*] The necropsies were performed by Drs. N. P. Hudson and J. H. Bauer, of the West African Yellow Fever Commission.

had been exposed for ten minutes in each case before they were replaced with the normal monkeys. The latter, in both these experiments, continued to have normal temperatures for twenty-one and sixteen days, respectively, and died of yellow fever when tested for susceptibility. They exhibited the typical pathological lesions of this disease.

Controls for Experiments II and III, consisting of ten and twenty-five infects from the respective lots, were drawn into catching tubes before they had quite finished feeding on the second or normal monkeys, so that there would be no doubt as to their having had the second or normal-blood feeding. An attempt was made to select only those that were previously observed to alight on the normal monkey with some blood already visible in their midguts. The control group of ten insects for Experiment II (sub-lot 138a) was tested for infectivity after twenty-one days, when six specimens failed to induce yellow fever in a normal monkey (*M. rhesus* E) by biting it. This animal was given a susceptibility test ten days later (by mistake the observation period was made hardly long enough). It died of yellow fever and showed typical pathological lesions at necropsy. Subsequently, another normal monkey (*M. rhesus* F) was bitten by eight mosquitos of the same control lot and showed no reaction, although later it had a group of suspicious high temperatures following the injection of a saline suspension of six of these insects. This animal proved insusceptible on retest with known infective blood-virus. After sixteen days the control in the third experiment (sub-lot 160a) induced fatal yellow fever in the original test rhesus (H).

The data for these three experiments with *A. aegypti* are presented in Table I.

CIMEX LECTULARIUS Linn.

A large-mouthed vial containing a stock lot of bed-bugs, including nine adults and approximately fifty larvae and nymphs, was first warmed next the skin to arouse in the insects a desire for food. The vial was then inverted directly on an infective monkey (*M. rhesus* I). After a short period the insects were disturbed and the vial was immediately transferred to a normal animal (*M. rhesus* J). Both

TABLE I.

Attempts at mechanical transmission of Yellow Fever with *Aedes aegypti*.

June 26, October 2, and December 4, 1928.

Interrupted feeding tests with mosquitos								Controls			
Experiment No.	Source rhesus	First fever	Lot No.	Estimated *A. aegypti* completing meal	Test rhesus	Results	Suscepti-bility	Sub-lot	Test rhesus	No. insects	Results
I	A	105·6 2nd day	78*	39	B	No reaction 17 days	Positive (see control)	78a	B	10	Died of Yellow Fever
II	C	105·4 3rd day	138*	60+	D	No reaction 21 days	Died of Yellow Fever	138a	E	6	No reaction. Susceptible on retest.
									I†	8	Non-fatal : resisted
										6	retest
III	G	105·1 3rd day	160*	100+	H	No reaction 16 days	Positive (see control)	160a	H	14	Died of Yellow Fever

* All original lots induced fatal yellow fever in subsequent monkeys (not listed) proving infectivity of source animals.
† *M. rhesus* F injected with saline emulsion of 6 insects 13 days after being bitten by 8 of same sub-lot. Developed suspicious fever and resisted retest with proved infective blood-virus.

animals had been immobilized as in the preceding experiments. In this manner four exposures of the normal animal, involving seven interrupted feedings of the bed-bugs on both animals, were allowed before all had finally become satiated. *M. rhesus* J remained normal and, after sixteen days, was tested for susceptibility. It developed a typical febrile course, beginning on the third day after injection of liver emulsion, known to be infective, but it recovered. The data are presented in Table II.

The control lot (165) of *A. aegypti* was allowed to feed on the source animal immediately after the bed-bugs had had their blood-meals ; a period of twenty-nine days transpired before this lot was tested for infectivity. The test animal, rhesus K, died after a typical course of yellow fever.

TABLE II.

Attempts at mechanical transmission of Yellow Fever with *Cimex lectularius*.

December 30, 1928.

	Interrupted feeding tests with bedbugs							Control with *A. aegypti*		
Experi-ment No.	Source rhesus	First fever	No. insects	Test rhesus	No. of mechanical exposures	Results	Suscepti-bility	Lot No.	Test rhesus	Results
IV	I	104·7 2nd day	9 adults 50 im-mature	J	4	No reaction	Fever: recovered	165	K	Died of Yellow Fever

DISCUSSION

A batch of normal *A. aegypti*, which is at least three days old and has been moisture-starved for about twenty-four hours, usually attacks a monkey with avidity within a few minutes of its insertion in the cage in which the mosquitos are confined. If weather conditions are good and the monkey is not too restive, the majority of the lot, if undisturbed, will have fed within the first ten minutes, and particularly during the second five minutes. The rapidity of withdrawal of blood depends upon the vascularity of the point of attack on the host. Those insects biting the arms and hands of an immobilized rhesus have in most instances been observed to become distended more quickly than those alighting on the shaven belly. Not infrequently the latter insects make several probes before a suitable vessel is tapped.

It is unfortunate that the small control lot in Experiment II did not give definitely positive results. An accident caused a delay in the removal of these control insects until after many of the lot had finished feeding on the second animal. Later, the first monkey (rhesus E) fed on by this control sub-lot (138a) was by mistake tested for susceptibility only ten days after exposure to the insects. The temperature record of the second rhesus (F) used for testing the infectivity of this same control lot of mosquitos by exposure to their bites and also by the injection of the ground-up bodies of the survivors leaves it doubtful whether or not the animal was immunized or

originally insusceptible. But the fact that the original mosquitos (Lot 138) from which these controls had been removed, later produced fatal disease in another monkey indicates that mechanical exposure occurred as definitely with the first test animal (D) employed in Experiment II as in the other two experiments in which *A. aegypti* were used.

Additional tests with *C. lectularius* were not carried out, since no temperatures of 104° F. or over were ever registered by test rhesus J during thirty-six days of morning and afternoon observations previous to its use in this experiment. It is conceivable that slight protection had been afforded by the bites of the bed-bugs, since Davis and Shannon (1929) have considered ' subinfective doses of virus ' to have been transmitted to *M. rhesus* by *A. aegypti* in their experiments ' with or without a slight rise in temperature.' (An adequate incubation period had elapsed before their insects bit the test-monkeys.) The pyrexial period following the test for susceptibility of rhesus J was so marked (maximum 106·6° F.), however, and so typical of the courses in other definite infections with yellow fever followed by recovery that this explanation is hardly probable, especially as no immunizing effect was apparent in the preceding experiments with mosquitos. It may be mentioned that in further experiments with bed-bugs, after appropriate intervals to allow for incubation the susceptibility of two normal animals was not reduced as a result of either the bites of these insects or the injection of their ground-up bodies. Both of the animals died of yellow fever when tested for susceptibility.

The possibility of the transfer of the virus of yellow fever on contaminated mouth-parts of insects during intermittent feeding thus appears to be remote.

Another angle of the problem suggests itself, the possibility of infection through regurgitation during feeding. Although these experiments would seem also to eliminate this possibility of mechanical transfer of the virus, the controversial status of this question seems to require discussion in regard to the mosquitos.

There is considerable divergence of opinion among investigators concerning the rôle played by the oesophageal diverticula in various species of mosquitos during the processes of ingestion and digestion of the blood-meal. Obviously, the chances for mechanical trans-

mission of yellow fever would be considerably enhanced were it true, as suggested by Schaudinn (1904) and others, that regurgitation from these crops takes place during the act of feeding, since blood has been found in these organs at such times by Nuttall and Shipley (1903), by Roy (1927), and by others. Roy found, in *Anopheles subpictus* and *A. stephensi*, that only the excess blood toward the end of the meal passed into the crops, whereas Nuttall and Shipley, experimenting with *Culex pipiens*, believed that these sacs acted as reservoirs during the ingestion of the meal. Falleroni's (1926) studies led him to believe that no regurgitation takes place, although Roy has stated that the wheal produced in three individuals by injection of emulsions of salivary glands is smaller than the wheal produced after bites, or after injection of the contents of the diverticula. Roy drew no conclusions with regard to this observation, although he says that ' absence of blood from the oesophagus led Christophers to suppose that regurgitation does not take place.'

It may be mentioned in support of the observations of several previous investigators that, during the act of feeding, experimental stock lots of *A. aegypti*, already containing some liquids such as sugar-solution, have been repeatedly observed to push the blood-meal on into the mid-intestine in varying amounts, depending on the relative development of the ovaries or the previous distention of the ventral crop in which the other fluids are stored. Moisture-fed females, when examined by transmitted light immediately after blood-meals, usually exhibit a sharp division between the blood-filled stomach and the clear liquid contents of the crop below and in front of the former. In several thousand females closely observed after feeding, the blood could never be definitely seen in the ventral crop, although partially developed ovaries might occasionally crowd the blood-filled stomach forward.

Dissections were made of seven female *A. aegypti* immediately after completion of their blood-meal and of ten that were interrupted when about half engorged. Five of the latter were killed immediately and the rest ten minutes after the interruption of their meal. The diverticula of all contained gas bubbles. Microscopic examination revealed traces of blood corpuscles only in the ventral crops of three and in the dorsal crops of two of those that had completed their feeding and in the dorsal crop of one of those allowed to live ten

minutes after the interrupted feeding; while the crops of all of those killed immediately after interruption contained blood in noticeable amounts, enough to give the crops a perceptible color under the low power of the binocular microscope. A small portion of the blood-meal, therefore, seems to pass through the diverticula during feeding, but is probably never detained there very long in *A. aegypti.* All of the seventeen insects dissected had emerged two days previously and had had no opportunity to imbibe liquids other than the water from which they had emerged, and that only at the time of emergence. The experiments (I to III) reported above support the opinion that regurgitation, if it occurs at all, does not effect mechanical transfer of the virus of yellow fever.

It may be mentioned that the common monkey louse, *Pedicinus* sp., was found to be capable of producing infection, when saline emulsions of 100 or more were injected into normal *M. rhesus* immediately after removal of the insects from infected animals.

Two deaths and one immunizing fever have been produced in normal monkeys in this way, preliminary to testing the possibilities of mechanical transfer of the virus by interrupted feedings of monkeys. Louse infections sufficiently heavy to permit the completion of these investigations were never again encountered nor could they be induced by close confinement of normal monkeys before the end of the writer's tour in West Africa. It seems reasonable to assume that chances for infection through monkey lice are at a minimum, however, since no cross-infections that could be laid to this cause were observed during the experimental handling of considerable numbers of infested monkeys separated only by the sides of the cages.

Minute amounts of unaltered blood could be distinctly seen in most of the lice injected in the experiments described above. Attempts to keep them away from the monkeys until this blood had thoroughly digested resulted in too rapid depletion of the stock for subsequent use.

SUMMARY AND CONCLUSIONS

A. aegypti and *C. lectularius* were tested for the power to transmit yellow fever virus mechanically from infected to normal *M. rhesus* monkeys. Negative results were obtained in three experiments

in which from thirty-nine to at least one hundred mosquitos were used. The mosquitos were allowed partial feedings on experimentally infected animals during the initial fever, were interrupted in their meal, and were then allowed to complete engorgement on normal susceptible monkeys. Two methods of interruption during blood-meals were employed.

Nine adult bed-bugs and about fifty in the larval and nymphal stages also failed to infect a normal rhesus during seven alternate transfers between the normal monkey and an infected one during one feeding.

The possibility of mechanical transfer of the virus by regurgitation during intermittent feeding of mosquitos, and of monkey lice (*Pedicinus* sp.) is discussed.

On the basis of the experiments here reported, the conclusion is reached that the chances for aggravation of yellow fever epidemics by mechanical transfer of the virus by insects that feed intermittently appear to be remote.

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A COMPARISON OF THE RELATIVE KILLING POWER OF CHLORINE AND CHLORAMINE ON SCHISTOSOME CERCARIAE OF THE HUMAN TYPE, TOGETHER WITH A NOTE ON THE RELATIVE STABILITIES OF CHLORINE AND CHLORAMINE

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It has long been established that the infective phase of the human Schistosome parasites cannot survive for a longer period than 48 hours unless they come in contact with the definite host. Although the larval stage of the worm is so short-lived, a large number of cercariae are produced by a single infected snail for some weeks, hence the importance of rendering the water supplies used for domestic purposes free from snails and cercariae. As the cercariae readily pass through the ordinary sand-filters some other method of rendering the water safe has to be devised if the supply is required for immediate use.

Various chemical agents, particularly chlorine, have been suggested as an active agent for killing the cercariae, but it appears from the results of such experiments as have been published that no general agreement as to the exact concentration of the chlorine necessary for killing the cercariae has yet been established.

In one publication emanating from the British War Office (1919)

it is stated that one part of available chlorine per million parts of water is effective in killing the cercariae. Manson-Bahr and Fairley (1920), in a series of experiments, found that cercariae of the human type were living and motile after 2½ hours in water containing 4 parts per million of chlorine. They conclude that ' one part per million of chlorine is the maximum that can be added without making the water quite unpalatable. Therefore, unless much greater quantities be added and some method of dechlorination afterwards employed, this method of purification of water infected with bilharzial cercariae must prove both unsafe and unsatisfactory.'

Blackmore (1928) found that one part per million of chlorine kills the cercariae of the human type in less than 30 minutes.

Owing to the great divergence in these observations and to the importance of this problem in a country where Schistosome infections are endemic, a series of experiments has been carried out on the effect of chlorine and chloramine on the cercariae of both varieties of the human Schistosome parasites.

EXPERIMENTAL WORK

Large numbers of Planorbis and Bullinus snails were examined and any of these which discharged cercariae resembling morphologically those described as Schistosome cercariae were selected.

The infected snails were thoroughly washed from any cercariae which might be adhering to their shells by placing them in funnels and washing them under running water for four to five minutes. They were then placed in fresh water and incubated at 37°C for twenty minutes then left at room temperature for another twenty minutes. It was hoped by this procedure that all the discharged cercariae would be quite fresh.

The amounts of available Cl_2 in bleaching powder and chloramine were carefully standardized and adjusted immediately before adding the cercariae. The cercariae and the disinfectants were placed in small cups and observed under the low power of the microscope.

An increase in the motility of the cercariae is observed following the immediate addition of the disinfectant; the motility then gradually ceases, the cercariae almost sink to the bottom of the cups,

nearly lying flat. In the higher dilutions the cercariae stay stationary in that position and the region of the anterior sucker undergoes convulsive movements alternately. This lasts for thirty or even forty-five minutes.

Evidence of death is taken as the cessation of any movement whatever, when the cercariae is observed under the low power of the microscope.

CHEMICAL INVESTIGATION

As a preliminary investigation the relative stabilities of chlorine and chloramine were determined, the chlorine solution being prepared from bleaching powder, the chloramine solution in the apparatus supplied by Messrs. The United Water Softeners Ltd. in accordance with the printed instructions supplied with the apparatus, namely :—

'Unscrew cap of chlor-sparklet apparatus, fill to 50 c.cm. mark with water and dissolve TWO of these tablets therein. The ammonium solution resulting is to be mixed with the chlorine solution in the bottle thus forming active chloramine.'

The stability experiments were undertaken with distilled, tap and raw Nile water, adding various dilutions of chlorine and chloramine. As a rule, the initial dose added was 1, 2, 3 and 4 parts per million.

As will be observed from the tabulated results it was found that the chloramine decomposed much more rapidly than the chlorine from bleaching powder. At the same time, the biological experiments showed that the lethal effects of chloramine on the cercariae was less than that of chlorine.

An interruption in the investigation occurred at this stage, but further determinations of the relative stabilities of chlorine and chloramine were made during August, September and October, 1929.

The later investigation confirmed the results of the preliminary tests. It had already been ascertained that when the chloramine solution was prepared according to the above mentioned method, between 30 and 40 per cent. of the available chlorine was immediately lost.

To maintain a solution that was relatively stable during the period of treating different waters—approximately ten to fifteen minutes—

the water in the apparatus was cooled to about 5°C and after addition to the solution of the ammonium compound, the bottle was kept in iced water.

In spite of this precaution the immediate loss of chlorine on mixing was never less than about 30 per cent.

It can, therefore, be concluded that chloramine solution prepared according to the instructions supplied, was very unstable, and that it rapidly decomposed in either concentrated or weak solutions even in the absence of substances in solution or suspension which would tend to deviate the chlorine.

Reference to the papers of Harold (1925-26) published in the Journal of the Royal Army Medical Corps, showed that poor concentrations of chloramine were obtained when the concentrations of chlorine and ammonia were high, and it was concluded that 25 parts per million of chlorine was the optimum concentration for practical purposes.

The yield of chloramine was then of the order of 90 per cent.

As this concentration is of the same order as that obtained in the kettle when the chloramine is prepared according to the instructions originally issued by the British Army for preparation of chloramine in a water cart, the latter procedure was now followed, with the exception that the ammonium chloride supplied by United Water Softeners Ltd. was used instead of the ammonium bicarbonate recommended by Major Harold.

The instructions just referred to were supplied with an earlier type of sparklet apparatus and are as follows :—

WATER CART METHOD

Mono-chloramine. Fill siphon to 1,250 c.cm. mark with water and discharge into it one chlorine bulb. SHAKE WELL. The siphon will then contain on an average 3 grammes of Cl_2. Fill up the kettle with water, crush a tablet of ammonium hydrogen carbonate, add it to the water in the kettle and stir well. 250 c.c. of the chlorine water is then added to the kettle (the cap of the siphon can be used to measure this), and the mixture is then well stirred.

Mono-chloramine is produced at once, and the contents of the kettle are emptied

into the water cart (110 gallons) which now contains 0·6 gramme of Cl_2 (0·5 gramme = 1 part per million in the cart). In this process there is no loss of available chlorine.

NOTE.—That the concentration of ammonia in the kettle is approximately 10 parts per million, and the concentration of chlorine 20 parts per million, also that the ammonia and chlorine must be added in the order stated.

It was concluded that in a large bulk of tap water containing bicarbonate the addition of ammonium chloride in equivalent molecular proportion instead of ammonium bicarbonate would make no perceptible difference and this supposition was justified by the results obtained.

To a jar containing about 20 litres of tap water 0·78 gramme of ammonium chloride (equivalent to 10 parts per million NH_3) in 25 litres of water, was dissolved, and 250 c.cm. of chlorine water, prepared in the apparatus and adjusted to 2,000 parts per million, was added by pouring down a rapid stream of tap water into the jar and shutting off the tap when the level reached the 25 litres mark. Thorough mixing was ensured by further stirring.

This solution contained 20 parts per million chloramine calculated in terms of the chlorine originally added.

The previous experiments, both as regards relative stability of chlorine and chloramine and as regards their comparative effect on cercariae, were now repeated, the chloramine being prepared by the method just outlined.

In the earlier series of experiments (Tables I–X) the available chlorine or chloramine was estimated colorimetrically by the O. Tolidine reagent. The estimation of residual chlorine presented difficulties in a turbid or coloured water, and therefore, in the examination of raw Nile water and water taken from the canal where the snails were found, the procedure adopted was to add a few crystals of potassium iodide to 355 c.cm. of the water, acidify with acetic acid, and titrate with N/100 thiosulphate using starch as indicator. Each c.cm. thiosulphate represents 1 part per million available chlorine. These figures were on some occasions checked side by side with the colorimetric method and were found to give results which agreed closely.

As will be observed from the tables the results obtained when the chloramine was prepared by the " water cart method " were very different from those obtained in July when the chloramine was prepared in the syphon.

THE EFFECT OF CHLORINE ON THE CERCARIAE OF THE HUMAN TYPE

The following experiments were carried out in July, 1929. In all of them cercariae of the *mansoni* type were used.

EXPERIMENT I.

Dilutions of the chlorine and the cercariae were carried out in tap water.

Source of chlorine : Bleaching powder.

Time of exposure : 15 minutes.

Cl_2 concentration		Result
1 in 1,000	... ...	... Died.
1 in 10,000 ...	...	... Died.
1 in 100,000	...	... Died
1 in 1,000,000	...	... Lived.
1 in 10,000,000	...	... Lived.
Control	...	... Living.

EXPERIMENT II.

Carried out exactly as above.

Cl_2 concentration		Result
1 in 1,000 ...	...	... Died.
1 in 10,000	...	... Died.
1 in 100,000	...	... Died.
1 in 1,000,000	...	... Lived.
1 in 10,000,000	...	... Lived.
Control	...	... Living.

EXPERIMENT III.

Dilutions of the chlorine and the cercariae were carried out in tap water.

Source of chlorine : Bleaching powder.

Cl_2 concentration	Result
5 parts per million...	... Died in 10 minutes.
4 parts per million...	... Died in 10 minutes.
3 parts per million...	... Died in 10 minutes.
2 parts per million...	... Died in 35 minutes.
1 part per million ...	... Died in $2\frac{1}{2}$ hours.
Control	... After $2\frac{1}{2}$ hours, few dead, majority living.

EXPERIMENT IV.

'Carried out exactly as No. III.

Cl_2 concentration	Result
5 parts per million...	... Died in 10 minutes.
4 parts per million...	... Died in 10 minutes.
3 parts per million...	... Died in 10 minutes.
2 parts per million...	... Died in 30 minutes.
1 part per million ...	... Died in $2\frac{3}{4}$ hours.
Control ...	... After $2\frac{3}{4}$ hours, few dead, majority living.

EXPERIMENT V.

Chlorine water used.

Dilutions of chlorine and cercariae were carried out in tap water.

Cl_2 concentration	Result
5 parts per million...	... Died in 10 minutes.
4 parts per million...	... Died in 15 minutes.
3 parts per million...	... Died in 25 minutes.
2 parts per million...	... Died in 1 hour.
1 part per million ...	After 7 hours in both the control and the
Control ...	Cl_2 cup all the cercariae were dead, and the majority disintegrated.

EXPERIMENT VI.

Carried out exactly as No. V.

Cl_2 concentration	Result
5 parts per million...	... Died in 10 minutes.
4 parts per million...	... Died in 10 minutes
3 parts per million...	... Died in 20 minutes.
2 parts per million...	... Died in 50 minutes.
1 part per million ...	... Not dead in 2 hours.
Control ...	... In 2 hours one or two cercariae were dead, rest living.

EXPERIMENT VII.

Chloramine was used in this experiment, and the dilutions were carried out in tap water.

Chloramine concentration	Result
6 parts per million...	... Died in 20 minutes.
5 parts per million...	... Died in 25 minutes.
4 parts per million...	... Died in 50 minutes.
3 parts per million...	... Died in $1\frac{1}{2}$ hours.
2 parts per million...	... Died in $2\frac{1}{2}$ hours.
1 part per million ...	... Not dead in 3 hours.
Control ...	... After $3\frac{1}{2}$ hours the cercariae in both the control and the chloramine were all dead, except one in each.

EXPERIMENT VIII.

This experiment was carried out exactly as No. VII.

Chloramine concentration	Result
6 parts per million...	... Died in 20 minutes.
5 parts per million...	... Died in 30 minutes.
4 parts per million...	... Died in 1 hour.
3 parts per million...	... Died in 1½ hours.
2 parts per million...	... Died in 2½ hours.
1 part per million ...	... Was not dead in 3½ hours.
Control ...	... The control contained only three living cercariae and the chloramine two, the rest in the control and the chloramine were all dead.

EXPERIMENT IX.

Dilutions were carried out in raw Nile water.

Source of chlorine : Bleaching powder.

Cl_2 concentration	Result
6 parts per million...	... Died in 10 minutes.
5 parts per million...	... Died in 15 minutes.
4 parts per million...	... Died in 30 minutes.
3 parts per million...	... Died in 1 hour.
2 parts per million... 1 part per million ...	After 3½ hours, two or three cercariae were living in both the control and the Cl_2, the rest were dead.

EXPERIMENT X.

Dilutions were carried out in raw Nile water.

Chloramine was used.

Chloramine concentration	Result
6 parts per million...	... Died in 20 minutes.
5 parts per million...	... Died in 30 minutes.
4 parts per million...	... Died in 30 minutes.
3 parts per million...	... Died in 1 hour.
2 parts per million... 1 part per million ...	Not dead in 3½ hours. Both the control and the one with the chloramine contained very few living cercariae, the rest were dead.

The following table (A) shows the results of the previous experiments :—

TABLE A.

Minimum Lethal Dose.

Type of cercariae	Time of contact									
	15 minutes		½ hour		1 hour		2 hours		2½ hours	
	NH$_2$ cl	B.P.	NH$_2$ cl	B.P.	NH$_2$ cl	B.P.	NH$_2$ cl	B.P.	NH$_2$ cl	B.P.
Tap water — M.	6	4	5	3	4	2	3	2	2	1
Tap water — M.	6	3	5	2	4	2	3	2	2	2
Raw Nile water — M.	6	5	4	4	3	3	3	3	3	3

N.B.—All the numbers refer to parts per million.　　M. = *mansoni*.

The following experiments were carried out in October and November, 1929, using both mansoni and haematobium cercariae.

The chloramine was prepared by the Chemical Section of the Public Health Laboratories according to the ' Water cart method.'

RESULTS

THE EFFECT OF CHLORINE AND CHLORAMINE ON THE CERCARIAE

EXPERIMENT I.

Type of cercariae : *haematobium*.

Dilutions carried out in tap water.

Chloramine concentration	Result
2 parts per million... ...	Died in 15 minutes.
1 part per million	Died in 30 minutes.
0·9 part per million ...	Died in 45 minutes.
0·8 part per million ...	Died in 45 minutes.
0·7 part per million ...	Died in 1 hour
0·6 part per million ...	Died in 1½ hours.
0·5 part per million ...	Died in 2½ hours.
0·4 part per million ...	After 3½ hours few cercariae dead, majority living.
0·3 part per million ...	
0·2 part per million ...	
Control	After 3½ hours one cercariae dead, rest living.

EXPERIMENT II.

Type of cercariae : *mansoni.*
Dilutions carried out in tap water.

Chloramine concentration		Result
2 parts per million...	...	Died in 15 minutes.
1 part per million ...	...	Died in 30 minutes.
0·9 part per million	...	Died in 45 minutes.
0·8 part per million	...	Died in 1$\frac{1}{2}$ hours.
0·7 part per million	...	Died in 2$\frac{1}{2}$ hours.
0·6 part per million	...	
0·5 part per million	...	After 3$\frac{1}{2}$ hours few cercariae dead,
0·4 part per million	...	majority living.
0·3 part per million	...	
0·2 part per million	...	
Control ...	...	After 3$\frac{1}{2}$ hours few cercariae dead, majority living.

EXPERIMENT III.

Type of cercariae : *haematobium.*
Dilutions carried out in tap water.

Chloramine concentration		Result
2 parts per million...	...	Died in 15 minutes.
1 part per million ...	...	Died in 1 hour.
0·9 part per million	...	Died in 2$\frac{1}{2}$ hours.
0·8 part per million	...	
0·7 part per million	...	
0·6 part per million	...	
0·5 part per million	...	After 3$\frac{1}{2}$ hours few cercariae dead, majority living.
0·4 part per million	...	
0·3 part per million	...	
0·2 part per million	...	
Control ...	...	After 3$\frac{1}{2}$ hours two cercariae dead, rest living.

EXPERIMENT IV.

Type of cercariae : *mansoni.*
Dilutions carried out in tap water.

Chloramine concentration		Result
2 parts per million...	...	Died in 15 minutes.
1 part per million ...	...	Died in 45 minutes.
0·9 part per million	...	Died in 2$\frac{1}{2}$ hours.
0·8 part per million	...	
0·7 part per million	...	
0·6 part per million	...	Few cercariae were dead in 3$\frac{1}{2}$ hours,
0·5 part per million	...	majority living.
0·4 part per million	...	
0·3 part per million	...	
0·2 part per million	...	
Control ...	...	After 3$\frac{1}{2}$ hours few cercariae were dead, majority living.

EXPERIMENT V.

Type of cercariae : *mansoni*.
Dilutions carried out in raw Nile water.

Chloramine concentration	Result
3 parts per million...	Died in 15 minutes.
2 parts per million...	Died in 45 minutes
1 part per million ...	Died in 2 hours.
0·9 part per million	
0·8 part per million	
0·7 part per million	
0·6 part per million	After 4 hours few cercariae dead,
0·5 part per million	majority living.
0·4 part per million	
0·3 part per million	
Control	After 4 hours all cercariae living.

EXPERIMENT VI.

Type of cercariae : *haematobium*.
Dilutions carried out in raw Nile water.

Chloramine concentration	Result
3 parts per million...	Died in 1 hour.
2 parts per million...	
1 part per million ...	
0·9 part per million	
0·8 part per million	After 3½ hours few cercariae dead,
0·7 part per million	majority living.
0·6 part per million	
0·5 part per million	
0·4 part per million	
0·3 part per million	
Control	After 3½ hours few cercariae dead, majority living.

EXPERIMENT VII.

Type of cercariae : *haematobium*.
Dilutions carried out in raw Nile water.

Chloramine concentration	Result
3 parts per million...	Died in 15 minutes.
2 parts per million...	Died in 30 minutes.
1 part per million ...	Died in 1½ hours.
0·9 part per million	Died in 2½ hours.
0·8 part per million	
0·7 part per million	
0·6 part per million	After 4 hours few cercariae dead,
0·5 part per million	majority living.
0·4 part per million	
0·3 part per million	
Control	After 4 hours one cercariae dead, rest living.

EXPERIMENT I.

Source of chlorine : Bleaching powder.

Type of cercariae : *haematobium.*

Dilutions carried out in tap water.

Cl_2 concentration		Result.
5 parts per million...	...	Died in 15 minutes.
4 parts per million...	...	Died in 15 minutes.
3 parts per million...	...	Died in 30 minutes.
2 parts per million...	...	Died in 45 minutes.
1 part per million ...	...	Died in 2¼ hours.
Control ...	...	After 2¼ hours all the cercariae were living.

EXPERIMENT II.

Source of chlorine : Bleaching powder.

Type of cercariae : *haematobium.*

Dilutions carried out in tap water.

Cl_2 concentration		Result
5 parts per million...	...	Died in 15 minutes.
4 parts per million...	...	Died in 15 minutes.
3 parts per million...	...	Died in 30 minutes.
2 parts per million...	...	Died in 1 hour.
1 part per million ...	...	Died in 2½ hours.
Control ...	...	After 2½ hours all cercariae living.

EXPERIMENT III.

Source of chlorine : Bleaching powder.

Type of cercariae : *haematobium.*

Dilutions carried out in tap water.

Cl_2 concentration		Result
5 parts per million...	...	Died in 15 minutes.
4 parts per million...	...	Died in 15 minutes.
3 parts per million...	...	Died in 30 minutes
2 parts per million...	...	Died in 1 hour.
1 part per million ...	...	Died in 2¼ hours.
Control ...	...	After 2½ hours one cercariae dead, rest living.

Experiment IV.

Source of chlorine : Bleaching powder.

Type of cercariae : *haematobium.*

Dilutions carried out in raw Nile water.

Cl_2 concentration	Result
5 parts per million... ...	Died in 15 minutes.
4 parts per million... ...	Died in 30 minutes.
3 parts per million... ...	Died in 1 hour.
2 parts per million... ...⎱	After 3½ hours few cercariae were dead,
1 part per million ... ...⎰	majority living.
Control	After 3½ hours all cercariae were living.

Experiment V.

Source of chlorine : Bleaching powder.

Type of cercariae : *haematobium.*

Dilutions carried out in raw Nile water.

Cl_2 concentration	Result
5 parts per million... ...	Died in 15 minutes.
4 parts per million... ...	Died in 30 minutes.
3 parts per million... ...	Died in 1 hour.
2 parts per million... ...⎱	After 3½ hours few cercariae were dead,
1 part per million ... ...⎰	majority living.
Control	After 3½ hours few cercariae were dead, majority living.

Experiment VI.

Source of chlorine : Bleaching powder.

Type of cercariae : *haematobium.*

Dilutions carried out in raw Nile water.

Cl_2 concentration	Result
5 parts per million... ...	Died in 15 minutes.
4 parts per million... ...	Died in 30 minutes.
3 parts per million... ...	Died in 1 hour.
2 parts per million... ...⎱	After 3½ hours few cercariae were dead,
1 part per million ... ...⎰	majority living.
Control	After 3½ hours all cercariae were living.

EXPERIMENT I.

Source of chlorine : Bleaching powder.

Type of cercariae : *mansoni*.

Dilutions carried out in tap water.

Cl_2 concentration	Result
5 parts per million... ...	Died in 15 minutes.
4 parts per million... ...	Died in 15 minutes.
3 parts per million... ...	Died in 45 minutes.
2 parts per million... ...	Died in 1 hour.
1 part per million	In 2½ hours few cercariae living, majority dead, after 7 hours few cercariae living, majority dead, and disintegrated.
Control	After 7 hours majority living.

EXPERIMENT II.

Source of chlorine : Bleaching powder.

Type of cercariae : *mansoni*.

Dilutions carried out in raw Nile water.

Cl_2 concentration	Result
5 parts per million... ...	Died in 15 minutes.
4 parts per million... ...	Died in 30 minutes.
3 parts per million... ...	Died in 1 hour.
2 parts per million... ...⎱	After 3½ hours few cercariae were living,
1 part per million ... ...⎰	majority dead.
Control	In 3½ hours few cercariae dead, majority living.

EXPERIMENT III.

Source of chlorine : Bleaching powder.

Type of cercariae : *mansoni*.

Dilutions carried out in tap water.

Cl_2 concentration	Result
5 parts per million... ...	Died in 15 minutes.
4 parts per million... ...	Died in 15 minutes.
3 parts per million... ...	Died in 45 minutes.
2 parts per million... ...	Died in 1 hour.
1 part per million	Died in 2½ hours.
Control	After 2½ hours all living.

The following Table B shows the results of the previous experiments.

TABLE B.

Minimum Lethal Dose.

Type of cercariae		Time of contact									
		15 minutes		½ hour		1 hour		2 hours		2½ hours	
		NH₂ cl.	B.P.	NH₂ cl	B.P.	NH₂ cl	B.P.	NH₂ cl	B.P.	NH₂ cl	B.P.
Tap water	M.	2	3	1	2	0·9	2	0·8	2	0·7	2
	M.	2	4	2	3	1	2	1	2	0·9	1
	H.	2	4	1	3	0·7	2	0·6	2	0·5	2
	H.	2	4	2	3	1	2	1	2	0·9	1
Raw Nile water	M.	3	5	3	4	2	3	1	3	1	3
	H.	3	5	2	4	2	3	1	3	0·9	3
	H.	0	5	0	4	3	3	3	3	3	3

N.B.—All the numbers refer to parts per million.   M. = Mansoni   H. = Haematobium.

TABLE I.

Bleaching Powder in tap water.

Initial dose calculated as chlorine in parts per million	Time of contact								
	½ hour			1 hour			1½ hours		
	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine
1	0·7	0·2	0·1	0·55	0·3	0·15	0·35	0·5	0·15
2	1·3	0·5	0·2	1·0	0·8	0·2	0·9	0·9	0·2
3	(2·5)	(0·3)	0·2	2·0	0·8	0·2	1·8	1·0	0·2
4	3·0	0·8	0·2	2·8	1·0	0·2	2·6	1·2	0·2

TABLE II.

Chloramine in tap water.

Initial dose calculated as chlorine in parts per million	Time of contact								
	½ hour			1 hour			1½ hours		
	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine
1	0·5	0·2	0·3	0·4	0·2	0·4	0·3	0·3	0·4
2	0·9	0·4	0·7	0·7	0·5	0·8	0·6	0·5	0·9
3	1·3	0·7	1·0	1·0	0·8	1·2	0·9	0·8	1·3
4	2·0	0·8	1·2	1·4	1·1	1·5	1·2	1·1	1·7

TABLE III.

Bleaching powder and chloramine in unfiltered Nile water.

Dose (calculated as chlorine)	2 parts per million		4 parts per million		6 parts per million	
Time	Bleach	Chloramine	Bleach	Chloramine	Bleach	Chloramine
½ hour	Trace	Nil	Present	Big trace	Present	Less than 4 P.P.M. Bleach
1 hour	Small trace	Nil	Present	Trace	Present	Less than 4 P.P.M. Bleach
1½ hours	Nil	Nil	Present	Small trace	Present	Less than 4 P.P.M. Bleach

Owing to the turbidity of the water, the residual chlorine could only be roughly judged.

TABLE IV.

Bleaching powder in tap water.

Initial dose calculated as chlorine in parts per million	Time of contact											
	½ hour			1 hour			1½ hours			2 hours		
	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine
1	0·6	0·4	0	0·5	0·5	0	0·45	0·55	0	0·4	0·6	0
2	1·4	0·5	0·1	1·2	0·7	0·1	1·1	0·6	0·3	1·0	0·6	0·4
3	2·2	0·8	0	2·1	0·9	0	1·9	0·8	0·3	1·8	0·9	0·3
4	3·2	0·8	0	3·2	0·8	0	3·0	1·0	0	2·8	1·0	0·2

TABLE V.

Chloramine in tap water.

Initial dose calculated as chlorine in parts per million	Time of contact								
	½ hour			1 hour			1½ hours		
	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine
1	0·4	0	0·6	0·4	0	0·6	0·3	0·05	0·65
2	0·7	0·1	1·2	0·7	0·2	1·1	0·5	0·2	1·3
3	1·0	0·5	1·5	0·9	0·1	2·0	0·75	0·25	2·0
4	1·2	0·2	2·6	0·9	0·5	2·6	0·9	0·3	2·8

Table VI.

Bleaching powder and Chloramine in unfiltered Nile water.

Dose calculated as chlorine.

		3 parts per million		4 parts per million		5 parts per million		6 parts per million		7 parts per million	
		Bleach	Chloramine	Bleach	Chloramine	Bleach	Chloramine	Bleach	Chloramine	Bleach	Chloramine
¾ hour	O.Tolidine	grey	grey	violet grey	grey	brown	grey brown	yellow brown	brown	yellow brown	brown
	Starch iodide	pale green	grey	blue green	green	blue	blue green	deep blue	deeper	intense blue	blue
1 hour	O.Tolidine	grey	grey	grey	grey	violet grey	grey	brown	brown grey	brown	brown grey
	Starch iodide	Nil	grey green	pale green	green	blue	blue green	deep blue	dark blue green	intense blue	blue
1¾ hours	O.Tolidine	grey	grey	grey	grey	grey	grey	violet grey	grey brown	brown grey	grey brown
	Starch iodide	Nil	greenish	Nil	blue green	green	pale blue	blue	blue	strong blue	stronger blue
3 hours	Starch iodide	Nil	grey	Nil	green	Nil	green blue	grey	blue	blue	blue

The above colours were given in addition to O. Tolidine reagent which gives an orange yellow colour with solution containing chlorine.

A blank test on raw Nile water gave a grey colour.

A brown or yellow brown was regarded as visible evidence of the presence of free chlorine but solutions which were blue grey and violet grey on addition of this reagent gave positive starch-iodide reactions for chlorine.

TABLE VII.

Chloramine.

Initial dose calculated as Chlorine in parts per million		Time of contact					
		¼ hour		1 hour		2 hours	
		Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine
1	Distilled water	0·4	0·6	0·4 Nil	0.6	0·3 Nil	0·7
	Tap water	0·5	0·5	0·4	0.6	0·35 Nil	0·65
2	Distilled water	0·8	1·2	0·6 V. pale blue	1.4	0·5 pale blue	1·5
	Tap water	0·55	1·45	0·5	1.5	0·5 trace	1·5
4	Distilled water	1·8	2·2	1·1 blue	2.9	1·2 deep blue	2·8
	Tap water	0 green	0	1·2	2.8	1·0 pale	3·0
6	Distilled water	2·5	3·5	1·6 deep blue	4.4	1·6 deep blue	4·4
	Tap water	0 green	0	1·6	4.4	1·0 blue	5·0

N.B.—None of these results, using O. Tolidine is exact as the colour was always green. The blue colour refers to the use of starch iodide indicator.

TABLE VIII.

Chlorine (from chlorine bulb) in tap water.

Initial dose calculated as chlorine in parts per million	Time of contact					
	¼ hour		1 hour		2 hours	
	Residual chlorine	Absorbed and decomposed chlorine	Residual chlorine	Absorbed and decomposed chlorine	Residual chlorine	Absorbed and decomposed chlorine
1	0·5	0·50	0·55	0·45	0·6	0·40
2	1·4	0·6	1·4	0·6	1·4	0·6
4	3·6	0·4	3·2	0·8	3·2	0·8
6	4·75	1·25	5·0	1·0	4·0	2·0

TABLE IX.

Chloramine solution in tap water.

Initial dose calculated as chlorine in parts per million	Time of contact											
	¼ hour			1 hour			1½ hours			2 hours		
	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine	Residual chlorine	Absorbed chlorine	Decomposed chlorine
1	0·45	0·35	0·2	0·45	0·3	0·25	0·4	0·2	0·4	0·45	0·2	0·35
2	1·0	0·5	0·5	0·7	0	0	0·9	0·3	0·8	0·7	0·7	0·6
3	1·6	0·6	0·8	1·5	0·6	0·9	1·0	0	0	1·2	0·4	1·4
4	2·0	0·8	1·2	1·4	1·4	1·2	1·4	0·8	1·8	1·2	0·8	2·0

TABLE X.

Chloramine and Bleaching powder in raw Nile water.

Initial dose calculated as chlorine in parts per million	Time of contact											
	¼ hour				1 hour				2 hours			
	Chloramine		Bleaching Powder		Chloramine		Bleaching Powder		Chloramine		Bleaching Powder	
	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine
¼	0·4	0·1	0	0	0·5	0	0	0	0·4	0·1	0	0
1	0·9	0·1	0·4	0·6	0·8	0·2	0·3	0·7	0·8	0·2	0·3	0·7
2	1·7	0·3	0·8	1·2	1·6	0·4	0·8	1·2	1·6	0·4	0·6	1·4
3	0	0	1·8	1·2	0	0	1·4	1·6	0	0	1·3	1·7
4	3·4	0·6	2·4	1·6	3·4	0·6	2·3	1·7	3·05	0·95	1·9	2·1
6	0	0	4·3	1·7	0	0	3·8	2·2	0	0	3·3	2·7

TABLE XI.

Chloramine and Bleaching Powder in canal water.

Initial dose calculated as chlorine in parts per million	Time of contact							
	¼ hour				2 hours			
	Chloramine		Bleaching Powder		Chloramine		Bleaching Powder	
	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine
¼	0·25	0·25	0	0	0·2	0·3	0	0
1	0·7	0·3	0·35	0·65	0·65	0·35	0·4	0·6
2	1·8	0·2	0·65	1·35	1·5	0·5	0·45	1·55
4	0	0	1·1	2·9	0	0	0·7	3·3

Initial dose calculated as chloride originally added (parts per million)	Time of contact					
	⅔ hour		1⅔ hours		2⅔ hours	
	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine	Residual chlorine	Absorbed chlorine
½	0·5	0	0·5	Nil	0·5	Nil
1	0·95	0·05	0·95	0·05	0·9	0·1
2	1·9	0·1	1·9	0·1	1·8	0·2
3	2·8	0·2	2·8	0·2	2·7	0·3
4	3·8	0·2	3·8	0·2	3·6	0·4

CONCLUSIONS

1. Both *S. mansoni* and *S. haematobium* cercariae are susceptible to the action of chlorine and chloramine.

2. One part per million available chlorine will kill the cercariae in two and a half to three hours in filtered water.

3. Chloramine, when prepared in the apparatus supplied by Messrs. United Water Softeners Ltd., and in accordance with the printed instructions which they supply, is less effective than chlorine in killing the cercariae.

4. Chloramine when prepared according to the "Water cart method" (Tables X–XII chemical) is more effective in killing the cercariae than bleaching powder. One part per million will kill the cercariae in one hour in filtered water.

5. Chloramine prepared by admixture of the ammonium compound and the chlorine solution in syphon was found to be far

less stable than chlorine prepared from bleaching powder or from chlorine bulb (Tables I–IX), whereas with chloramine prepared by the method used in the later experiments (Tables X–XII) the reverse is the case. The stability of chloramine was demonstrated particularly in its resistance to absorption by matter in suspension and solution. Absorption by tap water is only slight but is higher in both raw Nile water and canal water.

The absorption increases with the dose and also with the time of contact. The amount remaining after two hours contact is roughly three-quarters of the dose in raw Nile water and rather less in canal water. The amount of residual chlorine when bleaching powder is used is distinctly less, i.e., about half the dose in raw Nile water and still less in canal water after two hours contact.

6. Inquiries from different water-works around Cairo shows that the average time between the discharge of the water from the filters till it is received by the consumer in Cairo water-works is one hour, in Maladi one or two hours and in Giza two and a half hours. Therefore, chloramine can be used in the concentration of one part per million with practical safety for Bilharziasis. If the bleaching powder is employed in the concentration of one part per million some way of storing the water for about four hours has to be used before it is delivered to the consumer. This end may also be achieved by employing about three parts per million of chlorine from bleaching powder with subsequent dechlorination after one hour.

N.B.—Except in the case of Table XII the chloramine was prepared in the syphon supplied by the " United Water, Softeners Ltd."

In Tables X, XI and XII the chloramine was prepared in a dilute solution.

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ANTI-VARIOLOUS MEASURES IN PALESTINE

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I. INTRODUCTION

(*a*) GEOGRAPHICAL.

Palestine, characterised by diversity of climate and of physical features, is, according to the description of Buxton (1924), ' pushed out into the desert as if it were an outpost of Europe and Western Asia.' Bounded on the north by French Mandated Syria, and on the west by the Mediterranean Sea, it is separated on the south from Egypt and Hejaz territory by a line running from Rafa on the Mediterranean to Taba at the head of the Gulf of Akaba and then north-east, while its eastern boundary, following approximately the River Jordan and its lakes, marches with the Emirate of Transjordan. A country with such geographical connections and its consequent exposure on all frontiers to invasion by infectious disease is obviously more than ordinarily liable to become the seat of epidemic outbreaks, especially in view of its unique position on the great pilgrim routes.

(*b*) HISTORICAL.

It is difficult to hazard opinion as to when variola made its first appearance in this part of the world. The Hebrew terms employed in the Old Testament, for example, to denote great epidemics of pestilence, sudden in onset and fatal in effect, are of so indefinite and of so general a character that they might apply equally to cholera, plague or smallpox. The plague of boils, *shehin* (Exodus ix, 8-12), the disease by which the enemies of Jerusalem were to be destroyed (Zachariah xiv, 12) and the malady with

which Job was smitten ' from the sole of his foot unto his crown (Job ii, 7) have been identified, however, with true smallpox by many authorities.

Thus, regarding the plague of boils, Macalister considers it to have been smallpox, ' the blains, being explained by Talmudic writers as pustules, and an epidemic accompanied by boils and pustules being probably smallpox ' (Masterman, 1925). Certainly the first author to deal with the subject was Ali ibn Rabban of Tabaristan, the Persian province south of the Caspian Sea; in A.D. 850 he completed his Firdawsu'l-Hikmah, the ' Paradise of Wisdom,' which, in Part IV, Discourse 10, contains a chapter on smallpox; he was, moreover, one of the teachers of Abu Bakr Muhammad ibn Zakariyya of Ray, better known by the name Rhazes given to him by mediaeval Latinists. Undoubtedly the most distinguished of Moslem physicians, he was a prolific author, numbering among his works the first treatise on smallpox, written about A.D. 900, from his experience of the disease at Teheran, Persia, and at Baghdad. According to Browne (1921), one of the earliest books printed in Persia with movable types was a treatise on inoculation for smallpox, published in 1825 at Tabriz.

(c) INCIDENCE.

(1) *Pre-war occurrence.*

Precise information as to degree of incidence is unfortunately lacking. Smallpox is classified, however, as one of the common diseases of Palestine and Syria by Masterman (1925), who states that ' when it sweeps through the land it has a very high mortality.'

(2) *Post-war occurrence.*

In 1921, two fatal cases were reported; one was an unvaccinated child recently arrived at Ramleh from Beirut, Syria, where smallpox was prevalent, the other an adult at Dawaimeh, a village four hours' ride from Hebron. The latter was proved to be the result of contact with travellers from Transjordan, where the disease had assumed epidemic form and was probably not unconnected with the Moslem pilgrimage, ambulant cases having been observed at Mecca.

During 1922, smallpox appeared on two occasions. In Dawaimeh spread of infection was largely due to the inoculation by a villager

·of over 300 children from the original case. Of the number variolated, 120 children developed the disease and 10 died, or 8·3 per cent. ; of the 37 cases of true smallpox, which occurred among contacts of the initial case or of the inoculated children, 6 died, or 16·2 per cent. This outbreak, then, resulted in 158 cases with 16 deaths. Two cases were later notified in Jerusalem, one month after the cessation of the Dawaimeh epidemic, but apparently unconnected with it.

In 1923 a child succumbed to the disease shortly after its arrival in Tiberias ; infection in this instance was traced to Aleppo, where smallpox was then epidemic and where the family en route from Anatolia to Palestine had lodged several days. From this case seven others developed variola in virulent form and five died in all. Later in the year there was reported from Mejdal a single case, from which no secondary infections arose.

During the first two months of 1924, four cases occurred in the Jaffa District, the source of infection being a Yemenite Jew who, while in the incubation period of the disease, had entered the country by rail from Egypt. As this immigrant delayed seeking medical advice until the twelfth day of illness and as the 13 direct and 109 indirect contacts had meanwhile dispersed throughout the several colonies and settlements in the district, everything apparently favoured a considerable spread of the disease. The measures taken, however, resulted in but two of the direct contacts and one of the indirect developing smallpox. There was one death.

The prevalence of smallpox in Syria during spring and summer led to further outbreaks in Palestine during May and June. In May, two cases, one fatal, among the children of a labourer recently returned from Syria, occurred at Samakh, a village near the eastern boundary of Palestine and situated on one of the routes of entry. In June cases were reported from Gorabeh, a Bedouin encampment east of Lake Huleh, within a mile of the Syrian frontier.

Here the initial case was a Bedouin child who, after having been inoculated from a case of smallpox in a neighbouring Syrian village, returned to Gorabeh with the disease well advanced. From this case variolation was performed by the tribal medicine-men on a number of other children, seventeen ultimately showing symptoms of this artificially transmitted infection ; there were, however, no fatalities.

During 1925 there was no case of smallpox in Palestine, and this in spite of high incidence in Egypt and Syria.

In 1926 three cases of smallpox occurred, but there was no extension of the disease. Two cases in the Hebron District showed the source of infection to have been Amman, Transjordan; the origin of the case at Tiberias remained obscure but was probably Syria.

During 1927 only one case of the disease occurred, although smallpox was prevalent in Egypt and Iraq and epidemic in Syria. Infection had been contracted in Damascus where smallpox was rife. Patient arrived in Palestine on November 20, and was recognised in Jerusalem as suffering from smallpox only on December 3, some three days after the first appearance of the eruption and five days after his reaching the town. His being a pick-pocket rendered his movements and contacts impossible to trace, but the energetic measures taken prevented any further spread of the disease.

During the years 1928, 1929 and 1930, Palestine has been free from smallpox.

The following Table summarizes the foregoing :—

Year	No. of cases	No. of deaths	Origin of infection
1921	2	2	Syria, Transjordan.
1922	159	16	Transjordan, Variolation.
1923	9	5	Syria.
1924	23	2	Egypt, Syria, Variolation.
1925	0	0	—
1926	3	0	Transjordan.
1927	1	1	Syria.
1928	0	0	—
1929	0	0	—
1930	0	0	—
Total for 10 years ...	197	26	—

Analysis of these figures shows 60 cases of true smallpox (variola vera) and 137 cases of inoculated smallpox (variola inoculata) to have occurred in Palestine during the past decennium. It is of interest to observe how widely the death rates differed in the two forms of the disease ; thus the percentage mortality in variola vera was 26·6, in variola inoculata 7·3.

II. ANTIVARIOLOUS MEASURES

(a) UNDER THE TURKISH RULE.

In a historical note on pre-war conditions in Palestine the Director of Health in his Annual Report for 1921 states that there were ' few relics to be found of any pre-existing Government Health Service, and the testimony of pre-war residents confirmed the absence of any such organisation.' Masterman (1925) in an endorsement of these views draws attention to the fact that vaccination was but half-heartedly carried out even in the large towns and scarcely at all in the villages, while inoculation was still resorted to from time to time with disastrous results. The Turks, it is true, employed public vaccinators, but the estimate of medical men in practice here before the war that not more than 10 per cent. of the population were vaccinated proves how spasmodic and unavailing their prophylactic efforts must have been. Little wonder that Macalister (1925), referring to the then condition of Palestine, gave it as his opinion that ' the Holy Land is still, as it was in the Biblical period, a hot-bed of many diseases, which have lost none of their virulence during the post-Biblical centuries of misrule.' However, ' there is no reason to doubt ' he continues ' that given the enactment and enforcement of modern hygienic regulations, it might become one of the healthiest countries in the Eastern Mediterranean area.' In so far as freedom from smallpox is concerned, at least, that end has been already achieved.

(b) UNDER BRITISH ADMINISTRATION.

General procedure governing the control of communicable disease is, for all practical purposes, the same in Palestine as in England. Compulsory notification, under penalty, is secured by Ordinance, the duty devolving variously on medical attendant, on patient if adult, on head of house in which affected person is

resident, on ' mukhtar ' (elected head) of village or quarter, ' forthwith on becoming aware.' Procedure is facilitated by the issue of notification books to doctors, hospitals and ' mukhtars.' On receipt of notification domiciliary visits are paid and the necessary measures taken by the epidemic officers of the Department of Health. With regard to smallpox, special regulations issued by the Department detail instructions as to disposal of case—always to isolation or temporary hospital, disinfection of premises and clothing of patient, disinfection of persons and clothing of contacts, observation of contacts for 15 days, isolation of contacts when considered necessary, full investigation into outbreak with special regard to movements of patient for the previous 15 days. Should a case of smallpox occur, vaccination is energetically carried out on all immediate contacts and on all children in homes and schools nearby whether previously vaccinated or not ; should there be more than one case of the disease, vaccination is made to include all persons in the district affected. Fortunately in Palestine the legal enactments in regard to vaccination are ample. Thus, special powers are given to each District Commissioner, who may, by Public Notice, order all persons residing in his district or any part thereof to be vaccinated within a specified time, under penalty, unless such persons can produce to the Public Health Authorities proof of recent satisfactory vaccination. Further, vaccination of children is compulsory. Every child must be vaccinated within three months of birth unless its state of health demands temporary postponement. Control over such vaccination is exercised by means of the Birth Registers kept in each District Health Office, and proof of adequacy is afforded by the rarity of an unvaccinated child entering school to-day.

Additional routine measures include the vaccination of all immigrants arriving by the ports and of all Moslems making the annual pilgrimage to Mecca.

Fortunately for Palestine no recourse has yet been required to methods of persuasion and appeals to reason in favour of vaccination. The argumentative anti-vaccinator with his ill-advised propaganda finds no place in this country nor does the parent professing conscientious scruples with a view to his child's exemption. The generality of Palestinians still remember smallpox as Englishmen knew it in pre-Jennerian days and fully realise the value of vaccination as prophylactic and sure shield.

III. MODERN MEASURES IN APPLICATION

The comparative completeness of the measures of vaccination in force since 1918 has resulted in a country peculiarly well protected against smallpox. The estimated population of Palestine resident in towns and villages is about 800,000, and, according to the 1922 census, Bedu tribes augment this figure by 100,000. During the period 1921-1929 there were performed 862,705 vaccinations, of which number 613,251 were primary. Intensive vaccination of affected areas and the enforcement of stringent epidemic measures have in each outbreak been successful in preventing any considerable extension of the disease. Moreover the Dawaimeh epidemic of 1922, in which the principal cause of spread was the variolation of some 300 children, provided the Department with invaluable information regarding the calf-lymph then exclusively in use throughout Palestine. Manufactured at a private institute in Jerusalem, the vaccine proved wholly unable to cope with the emergency ; only 172 showed positive results among 2,734 vaccinations performed, and several children, apparently vaccinated successfully with this lymph, developed smallpox later in the epidemic.

Recourse was then made to Egypt, and an extensive use of fresh lymph of high potency, prepared at the Public Health Laboratories, Cairo, proved the turning point in the campaign, for not a single individual thus protected fell victim to the disease.

Apropos is an interesting observation made by Macqueen (1926), in connection with the immunity conferred by variolation. Twenty-seven children, who had been 'inoculated' and had developed eruption and symptoms of variola inoculata, were vaccinated with a reputable lymph twelve months after the occurrence, but out of the twenty-seven, successful insertion was obtained in 12 cases or 44 per cent.—a percentage surely much in excess of that likely to be obtained among twenty-seven children who had suffered from variola vera.

Control of the second outbreak of variola inoculata, which occurred in 1924, was easily exercised, on account of the isolated position of the affected encampment and by the performance of 7,000 vaccinations on a population recently transferred from Syria to Palestine and mostly unvaccinated.

During 1926, on account of the prevalence of smallpox in Egypt

and in adjacent territories, a campaign was organised in the spring of the year, having for its objects the vaccination of children belonging to the Bedouin tribes and the re-vaccination of school children and of all village children, those under five years of age showing definite proof of vaccination being alone excepted. A further measure was also brought into force at that time, the vaccination of all third-class passengers travelling by rail between Egypt and Palestine. In all, during 1926, there were 171,260 vaccinations performed, of which 79,853 were primary.

The succeeding years have abundantly proved Palestine to be a country in which vaccination is appreciated at its proper value. It will be recalled that the fatal case of smallpox in Jerusalem during December, 1927, was a pick-pocket, whose habits and movements rendered the tracing of contacts impossible. An intensive vaccination campaign was, therefore, launched ; three special vaccination stations were opened in the most populous centres of Jerusalem, calf lymph was supplied free of charge to hospitals and medical practitioners, active pro-vaccination propaganda formed part of the Department's scheme for coping with the situation. Response on the part of the inhabitants was remarkable ; within a month 34,295 vaccinations had been carried out, a figure equivalent to 50 per cent. of the town population. At the same time, in view of the epidemic prevailing in Iraq and the high incidence of smallpox in Egypt and Syria, opportunity was taken to urge vaccination in other parts of the country. As a result, 57,784 primary vaccinations and 40,788 re-vaccinations were performed.

Palestine, then, is well protected, due partly to the enthusiasm of its inhabitants for vaccination and partly to the Department's aim since 1918 at a universal and effectual vaccination.

IV. ANTIVARIOLOUS VACCINE

Anyone who has observed the disastrous effects of smallpox on a non-immune or badly-vaccinated village community, and has also noted the ease of control over an outbreak by means of a vaccine lymph of high potency, will surely advocate the necessity of a Public Health Department's keeping adequate stocks of reliable lymph, not only for routine vaccination requirements but to meet epidemic

needs. The obvious failure of a locally-produced lymph to control the Dawaimeh outbreak of 1922 had compelled the importation from Egypt of an excellent but somewhat expensive vaccine, and the desirability of a Government Lymph Establishment for Palestine had to be considered at an early date. The importation of calf-lymph is not only an uneconomic proposition in any country where suitable vaccinifers are to be obtained easily and at reasonable cost, but it frequently entails considerable loss of potency during transit. Since calves, best of all vaccinifers, are readily and cheaply procurable in Palestine and since the Department, in the interest of economy, pressed for sanction to manufacture its own requirements of calf-lymph, Government agreed to the erection of the premises and to the purchase of equipment necessary to carry out the undertaking. The Calf Lymph Establishment, opened in 1924 as an extension of the Central Laboratories and staffed by the then existing personnel of the Bacteriological Division, rendered the country, at small cost, independent of outside sources of supply. It was realised that the greatest likelihood of success in achieving a lymph stock of the desired potency would result from intensive production during the colder months of the year, January–April, and experience has shown that the work performed during that period assures a supply ample for the needs, routine and epidemic, of Palestine and Transjordan. Indeed, the stock of 600,000 doses, kept in reserve against unforeseen developments, has been called upon by the International Quarantine Board and by the Syrian Government, when their own ordinary sources of supply have failed.

(a) PRODUCTION OF LYMPH.

It is not proposed to detail the procedure followed here, except in so far as it differs to any extent from that described in text-books of Preventive Medicine and the like. The Establishment consists of the usual stable, attendant's quarters and various rooms used for the inoculation and scraping of calves, for the grinding of lymph pulp, for the filling of capillary tubes and for the performance of primary (control) vaccinations on children. It is built on the most hygienic lines and its close proximity to the main laboratories of the Department permits the requisite bacteriological investigation of lymph emulsions to be begun without delay. Only calves of from

8 to 10 months old are employed so as to obviate the necessity for milk-feeding; these animals, after one week's isolation and observation by a veterinary·officer, are transferred to the special stable, where they are accommodated in stalls, constructed of galvanised iron bars, and fitted near the anterior end with swing-gates of the same material. This arrangement, when the gates are closed, prevents the calf from licking the vaccinated surface, while allowing it full freedom in all other movements. Each animal is provided with a wooden floor batten, made movable to allow easy cleansing. During the process of vesicular development strong light is excluded from the stable by means of dark material suspended over the fly-proof doors and windows. Calves are prepared for operation in the usual way.

During the earlier period of the Establishment's existence, the method of vaccination was that of linear incisions. In the long axis of the abdomen a series of double incisions 3 to 4 inches in length was made by means of a moderately sharp scalpel, with the rounded back of which, previously dipped in seed vaccine, inoculation was effected. The double incisions were $\frac{1}{8}$th inch apart with an interval of about $\frac{3}{4}$ inch between each set. This method, at once tedious and time-consuming to the operator and most irksome to the calf, was further unsatisfactory from the point of view of lymph yield, since rarely could more than 3,000 doses per calf be thus obtained ; it was consequently abandoned in favour of the method now to be described. Over an area 4 inches by 4 inches are scratched parallel lines with the scalpel held sideways in the right hand, the left hand being used to put the skin on stretch. The lines 5 mm. apart extend from one margin of the area to the other and are of such a depth as just not to draw blood. These areas, numbering five to eight according to the size of the calf, are then smeared over with seed lymph. Vesiculation occurs in continuous and discrete lines, which do not coalesce. The yield of lymph is fairly large, an average-sized calf producing 7,000 to 8,000 doses, while the quality of lymph is excellent, its potency-index remaining at 100 throughout the weekly controls carried out in the children's vaccination room. After the areas have been suitably prepared, lymph is collected when five complete days have elapsed since vaccination ; very occasionally, however, an optimum development occurs somewhat earlier. On

conclusion of the ' scraping ' process the abdominal wall is powdered over with talc and the calf sent to the butcher. The animal is slaughtered in the presence of a veterinary officer who, after examination of carcass and viscera, including mediastinal and mesenteric glands, certifies as to the condition present. The tuberculin test is not applied.

The lymph is now weighed and ground ; during the grinding it is gradually diluted four times its weight with a solution of 50 per cent. glycerol in water to which 0·1 per cent. oil of cloves has been added. The diluent is carefully titrated to a reaction of pH7 before use. Complete trituration of the lymph is secured by a type of machine similar to that in use at the Lymph Establishment at Hendon, but fitted, in default of electric power, with 1-40th h.p. hot air engine. In Blaxall's words : ' the aim is to obtain a homogeneous emulsion with the pulp in a very fine state of division, and, if the triturating has been properly carried out, a loopful of the emulsion suspended in distilled water shows merely as a faint cloud, no definite particles being visible to the naked eye.'

After trituration, samples of the lymph emulsions are subjected to bacteriological tests identical with those required in England by the Regulations made under the Therapeutic Substances Act, 1925 ; immediately thereafter similar samples are placed in tubes, efficiently corked, and stored along with the bulk of the lymph in an environment of from −10°C. to −3°C. During storage, further bacteriological examinations are carried out from time to time and the number and nature of the organisms present carefully noted.

(*b*) COLD STORAGE.

That cold storage is essential for the preservation of lymph stocks requires no emphasis ; it is almost universally practised. When the question of storage first arose here, however, the only course available was to keep the lymph emulsions in direct contact with ice blocks until required for tubing. As the hot weather advanced, this method proved increasingly unsatisfactory, with the result that a solution to the storage problem became a matter of urgency if future lymph stocks were to retain their original potency.

Lack of municipal electric power together with Government's financial stringency precluded the erection of a refrigerating plant

and thus cold storage on the premises was an impossibility. The difficulties, however, were overcome by recourse to the following simple expedient which, for reasons of economy and efficiency alike, can with confidence be recommended to workers faced with a similar problem.

The lymph is placed in a specially designed ' container ' at the local ice factory. The interior of this galvanized-iron container, 1·50 m. high and 30 cm. square, is occupied by a light metal framework fitted with handles for easy removal and divided into six compartments. Each compartment is so constructed as to accommodate the wire baskets used to receive bottles of vaccine for storage. The container, closed and made waterproof by means of an accurately-fitting sheet-iron plate lined with rubber and held in position by hinged thumbscrews, is, after having been properly counterweighted, sunk in the brine tank of the ice factory and secured permanently by iron bands screwed to the wooden sides of the tank. The brine normally reaches to within six inches of the upper surface of the container, and as the manufacture of ice is carried on throughout the year, a temperature in the surrounding brine of from $-10°C$. to $-3°C$. is guaranteed, which ensures adequate cold storage. A small charge is made by the ice-manufacturer for the use of that part of the brine tank occupied by the container. A drawing of the container and its connections has been made on p. 539, which is self-explanatory.

It has been determined that lymph, produced in this country by the method described above and with an average initial flora of between 30 and 40 millions of colonies per 1 c.c., is rendered sterile in 23 days at room temperature (18°C.), in 15 weeks at the temperature of our cold storage, and in 4 days at incubator temperature (37°C.). The lymph virus, however, has been found to retain its potency after seven weeks' exposure to 18°C. ; after two and a half years in cold storage its virulence is inappreciably affected, while lymph is rendered wholly avirulent after one week at 37°C.
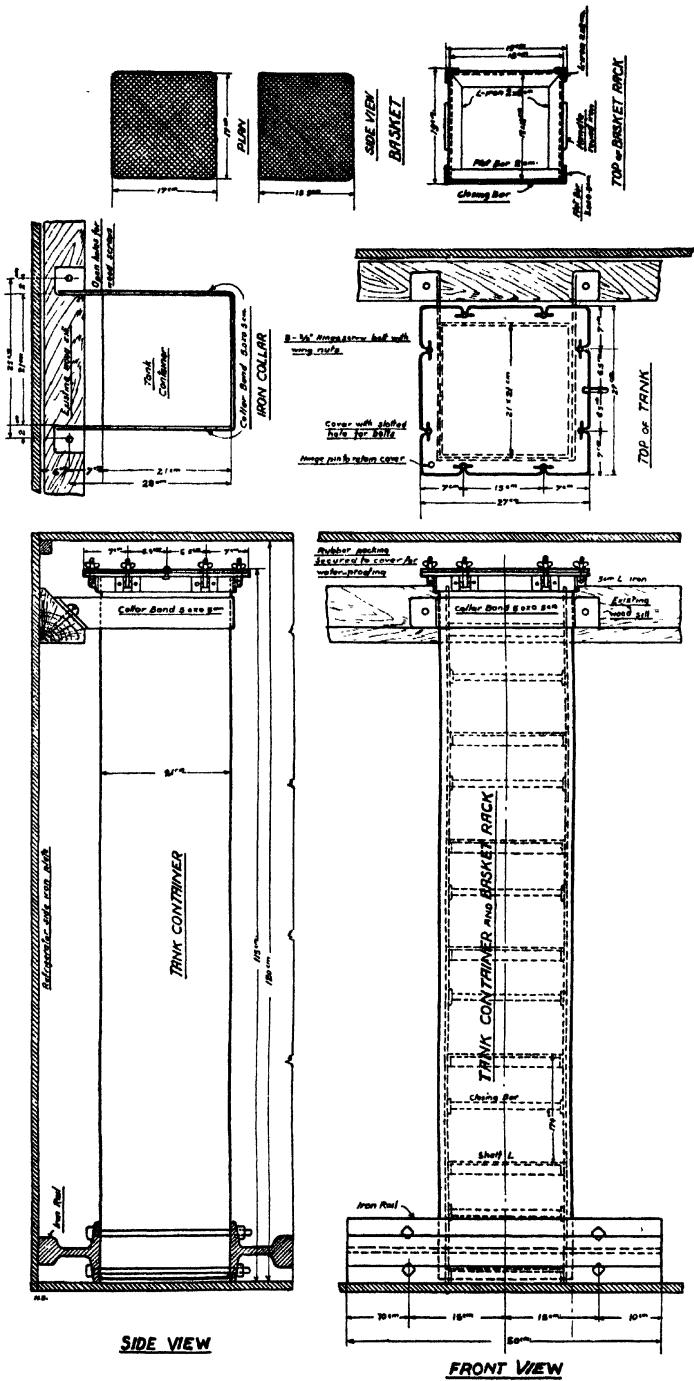
VACCINE REFRIGERATOR CONTAINER

SCALE 1:10

The rate of elimination of extraneous organisms during cold storage may be tabulated as follows :—

After storage period of	No. of colonies per 1 c.c.	After storage period of	No. of colonies per 1 c.c.
1 week	2,824,000	8 weeks	34,000
2 weeks	463,000	9 ,,	20,000
3 ,,	344,000	10 ,,	12,000
4 ,,	256,000	11 ,,	8,000
5 ,,	168,000	12 ,,	1,950
6 ,,	80,000	13 ,,	1,300
7 ,,	60,000	14 ,,	1,000 to Nil

It is, therefore, the practice here to issue lymph to vaccinators after a period of storage of not less than four months, and then only if proved bacteriologically free from extraneous organisms. The lymph is then filtered through gauze and tubed by Enterican's filling machine. Distribution is immediately effected to meet requirements, and instructions accompany each issue to the effect that, if not used within 14 days, the lymph must be returned to the Central Laboratories.

(c) SEED LYMPH.

The original supply of seed lymph was obtained from the Hendon Lymph Establishment, but its rapid deterioration in potency after a few passages on the calf necessitated its replacement by a further quantity from the same source. Since the arrival of this second consignment, regular periodic transfers on rabbits have succeeded in maintaining the potency of the seed lymph at a very high level. The rabbit is vaccinated in accordance with the late Dr. F. R. Blaxall's technique, viz. : the skin is very gently abraded and lymph rubbed in ; the result is a confluent eruption in which individual vesicles can with difficulty be distinguished ; the total yield is about 0·25 gramme, and here is collected on the 4th or 5th day and used to vaccinate a calf. The lymph from this calf is divided into three batches, one being used immediately for the next lot of calves, while the others are stored for future use.

This system of inoculating rabbits for the purpose of renewing stock may thus be graphically represented :—

```
                              Rabbit
                                |
                               Calf
                                |  .
        ┌───────────────────────┼───────────────────────┐
    Calf (1)                 Calf (2)                 Calf (3)
                                ┌───────────────────────┼───────────────────────┐
                            Calf (1)                 Calf (2)                 Calf (3)
                                                                                |
    ┌───────────────────────┼───────────────────────┼───────────────────────┘
  Rabbit                  Calf (1)                 Calf (2)                 Calf (3)
```
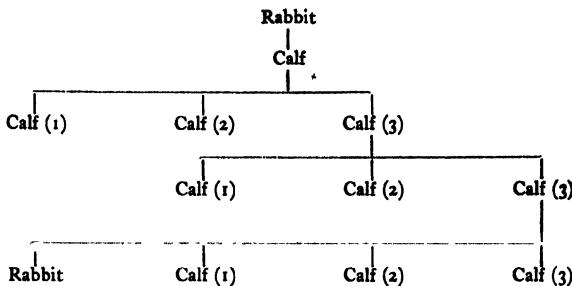
(*d*) TESTS FOR POTENCY.

Each batch of vaccine lymph, after the several procedures required for preparing it for issue have been completed, is tested for potency, so as to ensure its full activity at the time of issue. The unfiltered lymph sample under test is submitted to direct decimal dilution with N.S.S. and strengths of $1 : 10$, $1 : 100$, $1 : 1000$, $1 : 10,000$ are thereby obtained. Potency is gauged by the production of the characteristic lesions of vaccinia virus on application of the vaccine in these dilutions to the rabbit's skin. Ordinary rabbits of 1,500 grammes are employed. The fur over the vertebral column and to the extent of one inch on either side is removed and this area of shaven skin divided up into spaces with a moistened indelible pencil. Between the scapular regions and the pelvis five such areas are demarcated on either side. By means of a pipette one drop (0·05 c.c.) is delivered on to the centre of the first area on the right-hand side and well scratched in with a sharp needle. With fresh pipettes and needles one drop of each dilution is similarly applied to the succeeding spaces on the right side. On the left, identical operations are performed, but in this case the various dilutions are dropped on the shaven skin only, no scratching to ' insert ' the vaccine being practised. The last space on each side is reserved for the saline controls.

On the third day vaccinia reactions are normally to be observed in all four areas, on both sides of the vertebral column alike. These reactions become more marked on the fourth and fifth days and are fully as well developed on the left side as on the right, despite the

difference in mode of lymph application. (Proof has, however, also been afforded that the lymph manufactured here is invariably capable of producing vaccinia in the rabbit's skin in a dilution of 1 : 100,000.)

Tests on children are also carried out before issue. Infants numbering from 50 to 100 are brought weekly to the Establishment's vaccination hall, where the vaccinations are performed by the laboratory medical officers. Dilutions in this instance, however, are restricted to 1 : 100. These control vaccinations prove the lymph to have an efficiency value of 99 to 100 per cent. prior to distribution.

(e) RESULTS.

It must not be inferred that the original high success-rate is universally maintained in rural districts : such could not be expected, when the adverse conditions, under which many vaccinators have to work while on tour, are taken into consideration. The most practical method of ensuring the arrival of lymph, undiminished in potency, at outlying stations during the hot weather has been found to be at once the most simple and economical. Lymph tubes are surrounded by cotton-wool thoroughly saturated with water, and packed in boxes, of which the lid and sides are freely perforated. Experience has shown that the enclosed lymph tubes are kept quite cool by the evaporation of the moisture, the hotter and drier the air, the cooler the tubes.

Perhaps the quality of the lymph, on arrival at its many destinations throughout the country, may, however, be gauged best by reference to the following general statement which covers the last quinquennium.

Year	Total No. of vaccinations performed	Primary vaccinations		
		No. performed and observed	No. successful	Percentage of success.
1925	76,423	35,140	28,561	81·3
1926	171,260	69,965	60,760	86·8
1927	89,600	37,280	32,869	88·2
1928	98,572	51,287	44,106	86·0
1929	45,970	38,158	36,016	94·6

(f) COST OF PRODUCTION.

The production of smallpox lymph has entailed no extra expenditure here on laboratory personnel, for since the inception of the Establishment in 1924, it was undertaken by the staff of the bacteriological laboratory as part of their routine duties. The economic advantage to Government of such production at the Central Laboratories will be appreciated from the fact that during the first four months of 1930 the Calf Lymph Establishment manufactured 374,200 doses of vaccine at a net expenditure over revenue of £74 14s. The following summary explains the low cost :—

Number of calves vaccinated...	47
Number of calves successfully vaccinated	47
Number of grammes of pulp collected	1,871
Average number of grammes per calf	40
Number of doses manufactured	374,200
Purchase price of 47 calves	£142 16s.
Cost of forage, etc.	£47 10s.
Slaughter-house dues	£7 18s.
Sale price of 47 calves, meat and skins	£123 10s.
Net expenditure over revenue	£74 14s.
Cost of lymph production per dose manufactured... ...	0·048d.

V. SUMMARY

1. Palestine, from its geographical connexions and unique position on the great pilgrim routes, has been always peculiarly liable to invasion by epidemic diseases.

2. An account has been given of the incidence of smallpox in Palestine and of the anti-variolous measures in force before and after the Great War.

3. Reference has been made to the legal enactments, general and special epidemic procedure and the completeness of vaccination, which have together been instrumental in freeing Palestine from smallpox during the British Administration.

4. The thoroughness with which vaccination has been applied is reflected not only in a smallpox-free country but in the fact that no child on entering school is now found to be unvaccinated. Sharp contrast is afforded by present-day England ; whereas twenty years ago 75 per cent. of the population there were vaccinated in infancy, the percentage to-day vaccinated in early life is but 38.

Little wonder, then, that while Palestine is without smallpox, the disease is on the increase in England.

5.   Variolation has proved an unjustifiable and unsatisfactory immunising procedure.

6.   No case of post-vaccinal encephalitis has been encountered.

7.   It has been shown that any well-equipped bacteriological laboratory can, in addition to its routine work, undertake the successful manufacture of smallpox lymph without additional expert staff—an economy which must surely commend itself to any Administration.

8.   Lymph production has been discussed and a method of cold storage devised for establishments lacking electric power and/or financial independence.

9.   The remarkable enthusiasm of the inhabitants for vaccination and· the absence of anti-vaccinationists have played important rôles in ridding Palestine of smallpox.

VI. CONCLUSION

Palestine undoubtedly owes its present freedom from smallpox to its completeness of vaccination.   Admittedly other factors have been at work, and, to quote the words of Balfour and Scott (1924) ‘ even without vaccination we might have expected improvement, for, like many other diseases, smallpox flourishes best where dirt or overcrowding abounds. It may, however, be safely affirmed that without vaccination the malady would never have been fully controlled.’

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THE THIRD STAGE LARVA OF
STRONGYLUS EQUINUS

BY

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Little work has been done to clear up the life history of the strongyloid worm of the horse, many parts of which still remain unexplained. This neglect of an economically important parasite is probably due to the costliness of equines as experimental animals and the difficulty which there is in securing them free from infection with several species of strongyloid worms. Some of these are invariably present in ordinary animals more than a few weeks old, and add considerable difficulty to a study of the life history of any one particular species. The large size of the horse also discourages search for microscopic stages of the parasites and is doubtless the principal reason for the third stage larva of Strongylus having so long escaped observation. Fourth stage larvae have been described by Ihle and Van Oordt (1924 and 1925) and the presence of larvae in various organs and tissues has occasionally been reported. Nothing, however, is known of the route by which they travel nor of how they return to the intestine.

From time to time useful information has been derived from the experimental feeding of the infective stage of parasitic nematodes to small laboratory animals. Stewart (1917) was very successful in the use of rats and mice when working on the life history of *Ascaris lumbricoides*, as were Ransom and Cram (1921) working on the same subject. Mention of a few other records might be made, shewing the possibilities in this line of work. In a note Ransom and Raffeusperger (1920) recorded an observation on the development of an infective larva of *Arduenna strongylina* in a guinea-pig. On examination of the experimental animal eleven days after the administration of the infective feed one larva was found which had passed from the third to the fourth larval stage and grown to a size

of 5·8 mm., i.e., about three times its original size. Torres (1924) using guinea-pigs, shewed that pulmonary habronemiasis could be produced by placing larvae on the skin. Fielding (1927) reported that the larvae of *Oxyspirura mansoni* will live in the eyes of guinea-pigs, although they cannot reach this site unaided. In 1923 Ransom called attention to the use of the guinea-pig as an experimental host in work on the life history of nematode parasites and mentioned that *Haemonchus contortus* and ' certain horse strongyles ' will establish themselves in the intestinal wall. The aid of small laboratory animals has also occasionally been called in by workers on Ancylostomiasis.

An attempt was made by De Blieck and Baudet (1926) to obtain some information on the life history of the equine strongyles by using small laboratory animals. Their method was to feed large numbers of infective larvae to rats, mice, and guinea-pigs after a preliminary period of fasting, which in some cases was as long as twenty-four hours. In one experiment mice and rats received large feeds of larvae every twenty-four hours over a period of some days. The animals were killed for examination from one and a half to four hours after the last feed ; but although a few ensheathed and exsheathed larvae were recovered from the contents of the stomach and intestines and from the faeces, none were found in the gut wall, lungs, liver or elsewhere and the authors concluded that the infective larvae of equine strongyles pass through the lumen of the intestines of rats, mice, and guinea-pigs and do not penetrate into the body.

While working under the kind direction of Dr. M. C. Hall, in the United States Bureau of Animal Industry, the writer repeated this attempt to infect laboratory animals with strongyles and obtained rather better results than those previously reported. The work commenced in America was afterwards continued at the Veterinary Laboratory of the Ministry of Agriculture and Fisheries.

Preliminary experimental feeding of mice and guinea-pigs.

In a preliminary trial, a culture of infective larvae made from a mixture consisting of eggs of *Strongylus equinus*, *S. edentatus*, and *S. vulgaris* was administered *per os* in large doses to four mice and four young guinea-pigs (250 to 300 gms. weight). In the first mouse,

killed and examined two days after receiving the infective dose, a larva was observed in the liver, measuring 0·55 mm. long, and bearing three large bulbous appendages to the head, which had a general resemblance to the large lips of the adult Ascaris, but were proportionately larger. Nothing was found in any other organ, although a careful search was made in lungs, pancreas, spleen, kidneys, muscle, lymphatic glands, testes, salivary glands, wall of intestine and blood. Several ensheathed and exsheathed larvae, some of which were alive, were, however, recovered from the faeces. In two other mice examined three and five days respectively after the administration of the infective dose, nothing was found; but in a fourth, killed eight days after dosing a similar larva was found in the liver; there was, however, nothing seen in any other organ.

The first guinea-pig was killed five days after the administration of the first infective feed—a second feed having been given on the third day. The post-mortem examination of this animal gave most encouraging results; three of the larvae with trilobed head were found in the first two crush preparations of the liver. On examining the small lymphatic glands at the junction of the ileum and caecum, ninety-five of the same kind of larvae, arranged in two groups, were found.

Examination of other lymphatic glands, spleen, lung, wall of the small intestine near the ileo-caecal junction, and wall of the caecum, gave negative results. In the mucosa of the first part of the large colon, however, three larvae were found, in which the head appendages were absent. These larvae were longer, measuring 0 725 mm., more slender and more active than those which had previously been found in liver and lymphatic glands.

The other three guinea-pigs were not killed for examination until much later, but the results obtained in the first post-mortem examination of this experimental host were so promising that it was decided to make use of it for further feeding experiments.

Varying success of cultures of the three species of Strongylus.

The culture medium used was a mixture of equal parts of sterilized horses' faeces, and animal charcoal. In this mixture S. *equinus* was found to grow very readily, and in some cultures the number of larvae reaching the infective stage exceeded 1,500 for

every gravid female used in its preparation. Results from
S. edentatus were usually rather poor, and only on two occasions
did the final count of infective larvae exceed 500 per gravid female,
while *S. vulgaris* was quite the most difficult of the three to culture,
as a rule very few infective larvae, and sometimes none at all, were
recovered. It is not easy to account for these variations, and the
few changes in the method of culture which were tried during the
course of the experiments did not indicate the cause.

The general experience seems to be that *S. vulgaris* is the most,
and *S. equinus* the least, prevalent of the three species in the horse.
It is curious that the ease with which they may be cultured in the
charcoal-faeces medium should vary inversely as the occurrence
of the mature worms in the horse, and suggests that some other
differences may be looked far, possibly in their parasitic life.

Period required for development of the infective stage larva.

In order to determine this point some of the first cultures of
S. equinus were kept under careful observation at room temperature,
the earliest appearance of infective larvae noted was on the fifth day.
In two other cultures kept under observation they appeared on
the eighth and on the ninth day respectively. As the development
of some individual larvae is slower than others, cultures were not
used until at least two weeks old, in order to allow sufficient time
for the great majority of larvae to become ensheathed. In connection
with the rate of development of strongyle larvae, Martin (1911)
says that 45 to 60 days are necessary for the development of the
rhabditiform larva of *S. edentatus*. While according to Adelmann,
Baillet found that two to three weeks are necessary for *S. vulgaris*
to enter on the rhabditiform stage. De Blieck and Baudet (1926),
on the other hand, working with larvae which they thought to be
S. edentatus and *S. vulgaris*, found them to reach the infective
stage in from five to eight days. No attempts were made in the
series of experiments here recorded to determine the duration of
the first and second stage larvae of *S. edentatus* and *S. vulgaris*,
but observations made while separating larvae from the cultures by
Baermann's apparatus during the ordinary course of the work
suggest that the infective larvae of these two species develop as
quickly as that of *S. equinus*.

Method of procedure used in the feeding experiments

In the feeding experiments here described it was originally intended to use only larvae of *S. equinus* because of the ease with which they can be cultured, but the material could not always be obtained, and cultures of the two other species were sometimes used instead. For the experimental host young guinea-pigs of only 250 to 300 gms. weight were used because of the lower resistance generally shown by young animals to invasion by parasitic worms, and because their smaller size facilitated the search for microscopic larvae. The method followed was to give the animals a watery suspension of large numbers of the infective larvae by the mouth, after withholding food for twelve or sixteen hours. Then to kill them at varying periods after the experimental feed, and search for the larvae by means of crush preparation of tissues, with the idea of tracing their early wanderings in the body. The large mesenteric lymphatic glands, the liver and the wall of the colon were usually examined, while in some instances, where the experimental feed was an exceptionally large one or for some other reason the guinea-pig appeared to be particularly heavily infected, the lung, pancreas, peritoneum, wall of stomach, small intestine and caecum were also examined.

The following table gives a few details of the nature of the infective feed administered and the post-mortem findings in experimental guinea-pigs. It will be noted that in almost all cases the infective feed was of *S. equinus*, but that in some cases *S. edentatus* or *S. vulgaris* was given instead. The individual larvae ' A ' and ' B ' afterwards described were recovered from guinea-pigs which had received *S. equinus*, but larvae apparently identical with those were also found after the administration of infective feeds of the other two species.

Attempt to find larvae in the blood.

As much blood as possible was collected from guinea-pigs Nos. 1 and 2, by opening the chest and cutting into the ventricles of the still beating heart. These two guinea-pigs were killed ten hours and twenty-four hours respectively after the administration of the experimental feed. The blood was citrated, then laked with

No. of guinea-pig	Number of experimental feeds	Time between last feed and sacrifice of guinea-pig	Nature of the experimental feed administered	Findings			
				Liver	Lymphatic gland at junction of caecum and colon	Wall of large colon	Size of larvae
1	1	10 hours	Very large number of S. equinus	nil	nil	Numerous larva 'A'	0·61 × 0·012 mm.
2	1	24 hours	4,500 S. equinus	nil	1 larva 'B'	nil	
3	1	24 hours	S. equinus	nil	nil	nil	
4	1	48 hours	4,500 S. equinus	nil	nil	Several larva 'A'	
5	1	48 h urs o	Large number of S. equinus	nil	nil	nil	
6	1	48 hours	S. equinus	nil	1 larva 'B'	nil	0·70 mm.
7	1	3 days	Numerous S. equinus	3 larva 'B'	nil	nil	
8	2 in 3 days	3 days	Numerous mixed culture	4 larva 'B'	95 larva 'B'	3 larva 'A'	Larva 'A' 0·725 mm Larva 'B' from liver 0 430 mm.
9	1	3 days	S. equinus	nil	nil	nil	
10	1	4 days	12,000 S. edentatus	nil	11 larva 'B'	nil	
11	1	4 days	Few S. edentatus	nil	nil	nil	
12	1	4 days	S. equinus	nil	nil	nil	
13	1	6 days	5,000 S. vulgaris	nil	17 larva 'B'	nil	
14	1	6 days	7,000 S. edentatus	nil	nil	nil	
15	1	6 days	Many S. edentatus	1 larva 'B'	5 larva 'B'	2 larva 'B'	In lymphatic gland 0·43 mm. In liver 0·55 mm In colon wall 0·48 mm.
16	1	6 days	Few S. vulgaris	nil	nil	nil	
17	1	8 days	S. vulgaris	nil	nil	nil	
18	1	12 days	S. equinus	1 larva 'B' dead	1 larva 'B'	nil	In lymphatic gland 0·5 mm.
19	3 in 5 days	23 days	S. equinus	nil	nil	10 larva 'B' 1 larva 'A'	Larva 'A' 0·75 mm
20	1	23 days	Few S. edentatus	nil	2 larva 'B'	5 larva 'B'	
21	1	38 days	Mixed culture	1 larva 'C'	3 larva 'B' dead	5 larva 'B' dead	Larva 'C' 3·1 mm
22	2 in 5 days	96 days	S. edentatus	nil	nil	nil	
23	3 in 12 days	97 days	·S. equinus	nil	nil	nil	
24	3 in 12 days	97 days	S. equinus	nil	nil	nil	
25	3 in 12 days	99 days	S. vulgaris	nil	nil	nil	

NOTE.—Guinea-pig No. 7. Twenty white spots were found in the liver in three of which these larvae were found.
Guinea-pig. No 8. In the first two crush preparations of the liver three larva 'B' were found. The remainder of the liver was finely divided and an attempt made to separate the larvae by means of Baeman's apparatus, but only one was recovered.
Guinea-pig No. 13. Four white spots were seen in the liver, but nothing was found in them.
Guinea-pig No. 19. Two of the larva 'B' in the colon were surrounded by very definite cyst walls which could be seen with the unaided eye when the gut was pressed between two trichinoscope slides.
Guinea-pig No. 20. The diaphragmatic surface of the liver showed two wavy whitish lines presumably caused by large larvae wandering under the capsule. A number of small white spots were also found in the liver, some of which contained dead larva 'B.' The five larva 'B' found in the wall of the colon appeared to be dead, and were all surrounded by definite cyst walls.
Guinea-pig No. 21. The one larva 'C' was found in the omentum, but a wavy whitish track was seen on the diaphragmatic surface of the liver where it had presumably been wandering.

a large amount of water, and sedimented for twenty-four hours, the sediment being subsequently centrifugalised and examined for larvae, but with negative results.

Attempt to find larvae in the peritoneal cavity.

Guinea-pigs Nos. 1, 3, and 5 were examined in the following way. As soon as they were killed, i.e., ten, twenty-four and forty-eight hours respectively after the administration of the experimental feed, as much normal saline as the peritoneal cavity would hold was injected by means of a hypodermic needle and syringe. The abdomen was then worked about with the fingers, and the saline afterwards drawn off by the same means which had been used for its injection, and centrifugalised. No larvae, however, were recovered from the sediment.

Experiment to ascertain whether larvae penetrate the gut wall.

Two freshly killed guinea-pigs were taken. From one of these the whole bowel was removed, with complete mesentery attached. From the other, only the large intestine was removed, and the mesentery cut away close to the bowel wall. A large number of infective larvae were then introduced into the middle part of both colons by means of a long glass pipette. On withdrawing the pipette, the portion in which the larvae were situated was isolated by means of screw clips, in order to confine the larvae to one part and prevent them from escaping through the hole made by the pipette. The two experimental portions of bowel were then placed in normal saline at 37° C., the clamped ends being allowed to hang over the edges of the two vessels.

On examination after eighteen hours incubation three hundred and eight exsheathed larvae were recovered from the saline in which the gut with removed mesentery had been suspended ; while in the other vessel, where the mesentery with its contained blood and lymph vessels had not been cut, only one larva was found. The recovered larva in every way resembled larva ' A '.

Injection of larvae into the peritoneum.

It was thought that more larvae might establish themselves in the liver and develop there, if they were directly injected into the peritoneal cavity.

About nine thousand *S. edentatus* larvae were injected into the peritoneum of one guinea-pig, and in fourteen days an examination of the abdominal organs was made ; but three larvae ' B,' in the liver, were all that was recovered and the method was not followed up. The experiment showed, however, that passage through the intestine is not essential to the development of the larvae.

Injection of larvae beneath the skin.

This was done to ascertain whether the larvae can reach the liver and abdominal lymphatic glands through the circulation.

Larvae of *S. equinus* were injected subcutaneously into two guinea-pigs, which were examined after three and one hundred days respectively. In the experimental animal examined three days after inoculation only one larva ' B ' was recovered and this from the superficial inguinal lymphatic gland. In the other, examined after one hundred days, nothing could be found. No trace of larvae could be found at the seat of inoculation in either guinea-pig.

Description of parasitic larvae recovered from the guinea-pig.

Larva ' A.'

Three distinct larval forms were encountered in the examination of experimental guinea-pigs. What is regarded as the earliest of these was not met with in any part other than the wall of the large intestine. This larva, for the sake of convenience here called ' A ', is long and slender, measuring from 0·560 mm. to 0·750 mm. in length and only 0·020 mm. wide. It is much more flexible and active than the stage which follows, and is frequently found lying in four or five flexuous bends. The general morphology is similar to that of the ensheathed third stage larva, except that more detail can be made out in one or two places. At the head are the rudiments of the three large bulbous appendages which are so prominent a feature of the form to be described next. The lateral lines are frequently very distinct. The filariform oesophagus measures about 0·12 mm. in length. The intestine appears still to consist of sixteen dorsal and sixteen ventral cells and terminates in the anus situated 0·05 mm. to 0·06 mm. from the caudal extremity. The tail is blunt and does not taper evenly but shows a slight constriction between the middle and last third of its length. At about 0·06 mm.

from the anterior extremity the oesophagus is surrounded by the nerve ring, and at about 0·04 mm. further down the body the excretory pore opens on the ventral surface. What are believed to be the developing cephalic glands may be seen extending from about the level of the nerve ring to 0·08 mm. or 0·10 mm. beyond the end of the oesophagus. The genital primordeum is distinct as a group of specialised cells lying between the seventh and eighth cells of the intestine.

This ' A ' larva was found in four of the twenty-five experimental guinea-pigs and always in the wall of the large colon more or less close to the caecum. These four guinea-pigs were examined 10 hours, 2 days, 3 days, and 23 days respectively after the experimental feeding. In the first were found the largest number, and these were seen in their greatest concentration between half an inch and one and a half inches behind the junction of the caecum and colon. The mucous membrane appeared inflamed at this part. Smaller inflamed patches also occurred on the mucous membrane for a further six inches down the large colon, and microscopical examination showed each to be associated with a group of active larvae in the stage above described. No larvae were found in other parts of the intestine. Smaller numbers of larvae were found in the same site in the other three guinea-pigs ; and in the last one, examined twenty-three days after feeding, only one ' A ' larva was found among several ' B ' larvae.

Larva ' B.'

The next type of larva (here called larva ' B ') appears to be the stage which follows the one above described. It was found in the wall of the first part of the large colon, in the liver, and in a lymphatic gland situated near the ileocaecal junction ; three were also found in the portal lymphatic gland. They were most easily found, and almost invariably in the greatest numbers in the more ventral of the two small lymphatic glands situated near the junction of ileum and caecum. The large lymphatic gland more anteriorly situated in the mesentery was never found to contain larvae and presumably the ileocaecal gland in which they occur so frequently drains the wall of the first portion of the large colon, for which part of the intestinal wall they have a marked preference.

Larva 'B' usually shows a slightly smaller measurement of length than does the earlier stage larva 'A'; it is, however, stouter, and less flexible as is shown by the gradual curve in which it usually lies. The length varies between 0.43 mm. and 0·7 mm. The most striking feature of this larva, and by which, so far as is known to the writer, it may be distinguished from all other nematode larvae, is the presence of three large bulbous appendages of the head, resembling at first sight the three large lips of the mature Ascaris. Judging, however, from the position of the excretory pore, they are disposed so that one lies ventrally and two sub-dorsally so differing from the disposition of the lips of Ascaris. The nature of these swellings is not clear, and the only representative specimen of a later larval stage which was found in the experimental guinea-pigs shows no structure which appears to be in any way connected with them. It is, however, not improbable that they have some bearing on the early development of the mouth capsule, which is so prominent a feature in the genus Strongylus. Measurements of the internal parts compare very closely with those of the earlier stage previously described. They are, however, much more difficult to delineate, as the larva is usually more transparent, and is not easy to separate, without damage, from the tissues in which it is found. The cells of the intestine are still distinguishable, their number has not, however, been accurately determined. No appreciable development was observed in the genital primordeum but the head glands appear to have grown and now extend further forward.

Some stages which are regarded as intermediate between 'A' and 'B' were found in the wall of the large colon. A few larvae distinctly of the 'A' type presented more definite developments of the three head bulbs, while the body appeared less slender and tortuous than the one described. It was also noted that the head bulbs were smaller in some of the 'B' stage larvae than in others. These minor differences are suggestive of a gradual growth rather than the sudden change which is frequently seen after an ecdysis, and it is probable that both larvae 'A' and 'B' represent different phases in the development of one larval stage.

Beyond this stage only one larva has actually been recovered, although evidence of the existence of a similar one was found in a second guinea-pig. This larva 'C' was recovered from the
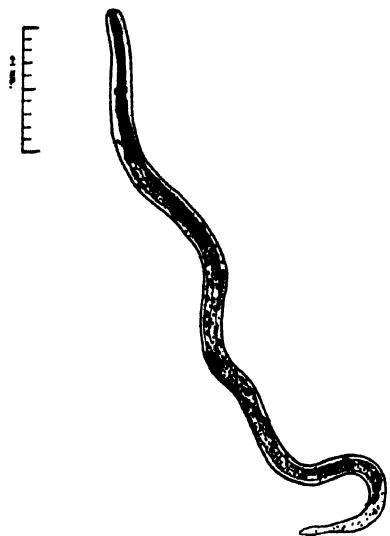
FIG. 1. Camera lucida drawing of *S. equinus* larva ' A ' (the young 3rd stage larva), from the first portion of the large colon of guinea-pig.
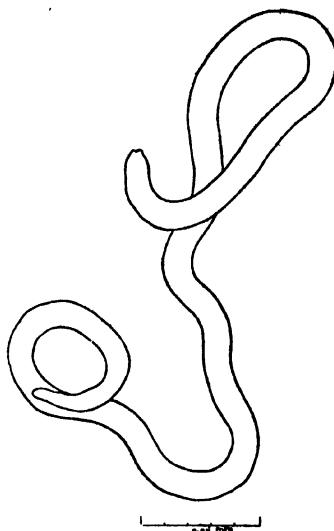
FIG. 2. Camera lucida drawing illustrating the flexible nature of larva ' A ' (the young 3rd stage larva).
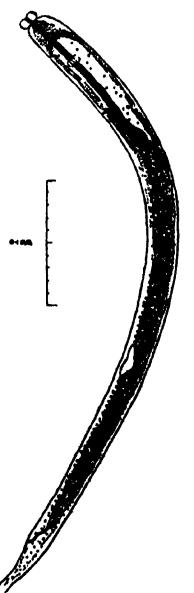
FIG. 3. Camera lucida drawing of larva ' B,' the 3rd stage larva, of *S. equinus* from the ileocaecal lymphatic gland of a guinea-pig.
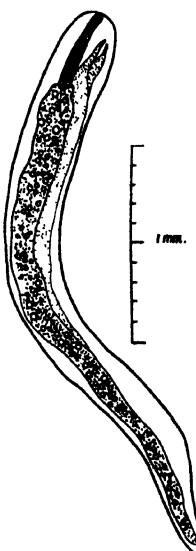
FIG. 4. Camera lucida drawing of larva ' C,' the 4th stage larva of *Strongylus* sp.

omentum, closely applied to the liver, in a guinea-pig killed thirty-eight days after the last infective feed. Evidence of its wandering under the liver capsule was seen in the form of a winding grey line on the diaphragmatic surface. Tracks of this kind may frequently be seen on the surface of the liver of infected horses, and fourth stage larvae may occasionally be found in them. The other experimental guinea-pig in which this lesion appeared was killed twenty-three days after the last feed, but although a careful search for the larvae was carried out it was unfortunately not recovered. The thirty-eight days old larva resembles in every way the fourth stage larva of *S. edentatus* described by Ihle and Van Oordt. It is, however, a little smaller than the smallest specimen they found in the horse, and measures 3·1 mm. in length and 0·3 mm. at its broadest point. The cuticle is covered with extremely fine striations. The mouth cavity is very shallow, leading almost directly into the oesophagus, and is surrounded by six small papillae. Surrounding the bottom of the mouth cavity is a ring which presents regular serrations on its anterior edge, probably representing an early stage of development of the mouth capsule. The excretory pore is also visible just behind the opening of the mouth. The oesophagus is comparatively short, measuring only 0·45 mm. in length. The anus is situated 0·05 mm. from the caudal extremity. It has not been possible to see much of the internal structures in this one specimen, but the head glands can be traced from a point 0·1 mm. from the head to mid-way between head and tail and occupy most of the space between these two points.

As the specific characters of the fourth stage larvae of the horse strongyles are not known, it is unfortunate that this larva was found in one of the only three guinea-pigs which received an infective feed of mixed larvae. Ihle and Van Oordt suppose the fourth stage larva they describe to be *S. edentatus* because of the large size of the cephalic glands. Thwaite mentions the same structures, which he describes as ' genital cells,' in fourth stage larvae found in experimentally-fed donkeys. These animals had received large doses of infective *S. equinus* larvae and because of this Thwaite assumes that the fourth stage larvae are of the same species. The available evidence, however, throws considerable doubt on this assumption as the only three larvae found which were sufficiently
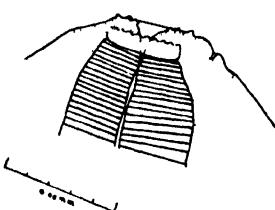
FIG 5   Camera lucida drawing of the head of the 4th stage larva of *Strongylus* sp.

FIG 6   Photograph of the diaphragmatic surface of a guinea-pig's liver showing the winding track left by the wandering 4th stage larva

mature to show known specific characters proved to be *S. edentatus*.

In experiments to be recorded later, infective *S. equinus* larvae were administered to a young foal which remained in the stable where it was born up to the end of the experiment. Some of the larvae recovered at the post-mortem examination were sufficiently mature to be recognised as *S. equinus* and it is highly probable that the fourth stage larvae recovered were of the same species. Examination showed these to possess the large cephalic glands which Ihle and Van Oordt had supposed to be a specific character of the fourth stage larva of *S. edentatus*.

This character, therefore, does not appear to hold, and the specific identity of Ihle and Van Oordt's fourth stage larva of *S. edentatus*, of Thwaite's fourth stage larva of *S. equinus*, and of the fourth stage larva recovered from this experimental guinea-pig is uncertain.

Discussion of Results.

From the findings above described it appears that when infective Strongylus larvae are swallowed by the guinea-pig many of them are able to free themselves from their sheaths. Of these a few are able to penetrate the mucous membrane of the large colon and appear to have a preference for the first inch or so of its length. After boring into the mucous membrane, one of three things may happen :—

Firstly, the larva may remain in the earliest stage ('A') without developing or causing any definite reaction in the gut wall. This condition is seen in guinea-pig No. 19, where one larva 'A' was found in the mucous membrane of the large colon twenty-three days after the administration of the last feed.

Secondly, the larva may develop to stage 'B' and still remain in the wall of the gut, where it can continue to live without increasing in size for a period up to twenty-three days. In some guinea-pigs there is a tendency for the formation of a cyst wall round larvae in this position.

Thirdly, the larva may pass through the gut wall and enter either the blood stream or the lymphatics. If it enters the lymphatics it usually finds its way to the more ventrally disposed of the two glands situated at the ileocaecal junction, which gland probably

receives the lymphatics from the first inches of the large colon. Whether larvae which reach this gland are able to migrate elsewhere is not known, but they have been found here alive up to twenty-three days, and dead up to thirty-eight days after the last infective feed.

Larvae entering the blood stream are carried to the liver where they remain and usually die before further development takes place, no evidence of their passing beyond this organ having been obtained. Attempts to find larvae in crush preparations of the lung have failed, as have attempts to recover them from the blood, and it is probable that those leaving the bowel by way of the blood stream rarely pass beyond the liver.

In connection with the time that larva 'A' and 'B' may remain dormant it is interesting to recall an observation of Scott's (1928) on hookworm larvae in rats, dogs and cats, where some individual larvae were found to remain undeveloped up to twenty-one days in the rat, thirty-three days in the dog, and forty-four days in the cat.

Occasional larvae which find their way to the liver are able to continue their development to a further stage—that of the fourth stage larva (larva 'C'). But of the large numbers given to guinea-pigs during the course of the experiments only two appear to have been able to reach this stage.

In a host so far removed from the natural one it is only those individual parasites possessing some special adaptive power which are able to survive, and the development of Strongylus larvae in the guinea-pig appears to depend chiefly upon their individual adaptability. The number of larvae which were able to penetrate the mucous membrane was a very small proportion of the number given to guinea-pigs, and although the majority of those which succeeded so far were able to reach stage 'B' only two were found which had developed beyond it. The reaction of the individual experimental host appears also to have some influence on larval development, evidence of which is derived from guinea-pigs 19 and 20, where the unusual formation of a definite cyst wall round each larva situated in the gut wall was observed.

The interpretation of results is complicated by the abnormal host-parasite relationship, and the question arises as to whether the larva which has here been described in two phases as 'A' and 'B'

is really the third stage larva of Strongylus, or some extraordinary form resulting from growth in so unnatural a host as the guinea-pig. An unsuccessful search has been made in the liver, and lymphatic glands of the large intestine of two foals to which heavy doses of infective larvae had been administered; but the comparatively enormous size of the natural host renders the search so much more difficult that this want of success is of no significance.

So far as can be ascertained no such change in the morphology of a nematode parasite, dependent upon its development in an unnatural host, is known to occur in any other species, and it is probable that the larva here described as ' B ' is the mature third stage larva of *Strongylus equinus* which may later be found in the liver, lymphatic glands of the colon and caecum, and perhaps also in the wall of this part of the intestine of the horse.

SUMMARY

1. In charcoal-faeces culture the infective larval stage of *S. equinus* may be reached in as short a time as five days.

2. Strongylus larva will live in guinea-pigs for as long a period as thirty-eight days and may develop as far as the fourth larval stage.

3. What is regarded as the third stage larva of Strongylus has been found in the liver, wall of the first part of the large colon, and some lymphatic glands of the experimentally infected guinea-pig.

4. In the guinea-pig, larvae do not penetrate the peritoneum but reach the liver by the blood stream, and the lymphatic glands by the lymphatic vessels.

5. A short description of the third larval stage of Strongylus is given. It may be distinguished from all known nematode larvae of other genera by the presence of three large bulbous appendages to the head.

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CONTRIBUTION TO THE STUDY OF EGYPTIAN SCHISTOSOMIASIS

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PLATES XIV-XVI

The fact that the schistosomes produce lesions mostly in the urinary and alimentary tracts justifies the publication of the following notes describing the pathology of rare bilharzial lesions outside the urinary and alimentary regions. The four cases described below show clearly the proliferative type of bilharzial lesion which may amount to actual tumour formation.

CASE No. 1. This is a case presenting sub-peritoneal papilloma due to *Schistosoma mansoni*.

The patient, H.A., a farmer 30 years old, came from a village near Cairo, and was admitted to the Qasr el Ainy Hospital on 13th November, 1929, and died on 9th December, 1929. The following were the clinical findings on admission.

He was very anaemic, emaciated, and had ascites ; he complained of loss of appetite and discomfort in epigastrium, and of passing blood and mucous in the stools. The liver was enlarged three fingers below costal margin and was smooth, the spleen was enlarged and was felt two and a half fingers below costal margin. The stools contained ova of *Schistosoma mansoni*. The urine contained albumin, granular and hyaline casts, and blood and pus, no ova were found in it. The blood count was as follows :—Total red cell count 3,612,000, the white blood cells were 7,400, Polymorphs 88 per cent., Lymphocytes 7 per cent., Hyaline 2 per cent., and Eosinophils 3 per cent.

At post-mortem, the left ventricle of the heart was hypertrophied, both lungs showed congestion and oedema at the bases. The liver

was enlarged and showed both periportal and diffuse cirrhosis, numerous *Schistosoma mansoni* worms were found in the blood of the portal vein. The peritoneal covering of both the under and upper surfaces of the organ was studded with whitish lustrous non-caseous nodules. The spleen was six times its normal size and its peritoneal surface was studded with nodules similar to those on the liver.

A moderate amount of clear fluid was found in the peritoneal cavity. The peritoneum over both small and large intestine, urinary bladder, and that of the omentum and mesentery was studded with closely set ivory-white flattened nodules similar to those on the liver. All the mesenteric glands were much enlarged, those at the root of the mesentery were matted together. On section the glands were harder in consistency than normal, non-caseous, and showed a typical sandy colour which to the experienced eye is suggestive of a schistosome affection of them. Some glands contained islands of calcification in the cut section. A scraping made from the cut section of these glands and examined at the moment, revealed their nature, as numerous lateral-spined ova were seen in every field.

The kidneys were congested, the ureters showed advanced deposits in the form of sandy patches and the mucosa of the urinary bladder showed a similar change.

Hanging from the peritoneal surface of the sigmoid colon, a bluish yellow, sausage-shaped tumour was found attached to the gut by a thick pedicle and measuring about 8 cms. in length by 2 cms. in diameter near the broad free end. It had a hard fibrous consistency, smooth even surface and a rusty white colour in section. The mucous membrane of the large intestine showed numerous papillomata.

Microscopically the sub-peritoneal papilloma described above showed diffuse distribution of lateral spined ova in various stages of degeneration and calcification embedded in a fibrous tissue ; the latter is either poor or rich in eosinophil cells according to its stage of development and also according to the condition of the ova situated in it ; the healthier looking ova are surrounded by numerous eosinophil cells and younger fibrous tissue, and the more degenerated and calcified ova are surrounded by more mature fibrous tissue with scanty or no eosinophil cell infiltration. This older fibrous tissue

is well described as fibroid or hyaline resembling very much the hyaline scar tissue around healing tuberculous lesions ; it is also poorly vascular. Neither the peritoneum over this papilloma nor that on the flat peritoneal nodules described above showed any proliferation in its endothelial layer ; this lesion is, therefore, a pure sub-peritoneal connective tissue lesion in contradistinction to another variety of peritoneal Schistosomiasis, in which the weight of the irritation falls on the endothelial elements of that membrane. This latter produces widespread nodular lesions of an ivory-white colour which show microscopically a typical picture of solid endothelioma with ova in each nodule. This last condition was described as diffuse bilharzial peritoneal endothelioma by my colleague Dr. M. F. Sorour.

The mesenteric glands in this case showed an especially heavy deposition of ova not only in the parenchyma but also in the sub-capsular connective tissue, so that on either side of the sub-capsular lymph-sinus ova are heavily deposited, on the one hand in the parenchyma of the gland and on the other in the very much thickened fibrous capsule. In the parenchyma of the glands, the ova are diffusely distributed producing a more or less diffuse tissue reaction. The elements of this tissue reaction differ with the age of the lesion ; in the younger ones, it is distinctly cellular, consisting of endotheloid and plasma cells with no eosinophil cells. In slightly older lesions, eosinophil cells are added to the above-mentioned elements and after the appearance of these a fibroid transformation starts in the cellular tissue which continues till complete solid fibrosis is established as seen in the very old lesions where the ova have also become calcified or destroyed by giant cells and disappeared. In such chronic lesions no eosinophil cells are to be seen.

The Spleen. A few ova were found deposited in the pulp with hardly any tissue reaction around them beyond a thin strip of giant cell nearly surrounding some of the ova. All the ova looked healthy and not degenerated ; in one ovum, however, a thickish zone was seen immediately under the shell which has taken up the haematoxylin stain well ; this always indicates calcification of the ova. The pulp of the spleen showed active proliferation of the pulp cells and a fair number of eosinophil cells amongst the pulp cellular elements.

The Liver showed advanced periportal and diffuse cirrhosis with many ova mostly healthy, and a few worms in the periportal tissue. Eosinophil cells were heavily deposited around or near some of the living ova simulating an eosinophil abscess.

The Lungs showed living ova with heavy infiltration of eosinophil cells around some of them ; worms in section were also seen in these organs.

The Large Intestine showed many ova in the mucosa and submucosa and some in the muscular coat ; ova were also present in the sub-peritoneal nodules described above which showed a microscopical picture similar to that of the pedunculated sigmoid papilloma.

Urinary Bladder showed a heavy deposit of ova in the mucosa and some in the submucosa, and also nodules under the peritoneum similar to those of the gut.

CASE No. 2. This is a case of multiple Schistosome tumours in the great omentum. The patient was admitted to the Qasr el Ainy Hospital suffering from an ordinary inguinal hernia which was not strangulated. At operation the contents of the hernial sac in the scrotum were found to be an omental tumour mass which was adherent to the hernial sac. This was dissected out and as much of the omentum as possible was removed with the tumour mass and resected and the rest of the operation was done in the usual way and the patient discharged cured. On examining the omentum a hard nodular mass about 9 by 4 by 2·5 cms. was found situated on the free border of the resected part of the great omentum. This mass on section showed several sandy nodules with dense white tissue surrounding them. In the rest of the great omentum were found several small nodular masses also hard and about the size of a date-stone, with the above-mentioned sandy colour on section. Scrapings from the pulp of these nodules showed lateral spined ova.

Microscopically, sections from these masses showed the two types of tissue reaction, viz., the diffuse type previously described, and the nodular type. In the nodular type, the ova are deposited singly or in small groups of two or three at most. The youngest ova are soon surrounded by many layers of typical endothelial cells frequently with the formation amongst them of huge giant cells immediately surrounding the ova or a little way from them. These

discrete tubercle-like nodules are seen embedded in a fibrous matrix. Up till this stage, no eosinophil cells are to be seen, but later on heavy eosinophil cell infiltration appears in the fibrous matrix surrounding the nodules. Contemporary with the appearance of the eosinophil cells, a fibroid transformation starts in the peripheral layers of the endothelioid cells of these discrete nodules, which continue concentrically inwards till these nodules become completely transformed into whorly layers of fibroid tissue. This fibroid transformation of the endothelioid nodules, immediately after the appearance of the eosinophil cells, is so often observed as to warrant attributing to this latter cell the stimulus to the commencement and completion of the fibroid transformation of the endothelial elements, this being a new function which Dr. M. F. Sorour attaches to the eosinophil cell. Microscopically schistosome worms were seen in section in some of these omental tumours.

CASE No. 3. This is a case of diffuse Schistosomiasis of the seminal vesicles and vasa deferentia.

The patient A. A., 30 years old, a dyer, came from a village near Cairo, and was admitted to the Qasr el Ainy Hospital on account of mitral disease on 31st March, 1930, and died on 3rd April, 1930. His urine was positive for *Schistosoma haematobium ;* the faeces were not examined.

At post-mortem examination the liver was found enlarged and fatty and schistosome worms were detected in the portal vein. The urinary bladder showed flat papillomata and sandy patches in its mucous membrane ; the deposit of ova was severer around the urethral orifice. The two ureters showed also sandy deposit.

Behind the urinary bladder, both seminal vesicles with their vasa deferentia were fused together in one mass which, with the prostate as a lower apex, formed an equilateral triangle with the base upwards and the two vasa deferentia coming from the two upper angles. The mass measured about 7 cms. from side to side by 3.5 cms. from above downwards and 2 cms. antero-posteriorly. Both vasa were thickened and cord-like, especially the right one. On cutting through this tumour mass, the nearly obliterated lumina of the seminal vesicles could be seen enclosed in rounded masses of a pale rusty colour embedded in a white fibrous matrix.

Microscopically, sections through the seminal vesicles show as

usual the lumina of the vesicles cut in different directions but much attenuated and embedded in a dense fibrous tissue heavily infiltrated with ova of the terminal spined variety, nearly all calcified. Some ova are deposited immediately below the mucosa of the vesicles while others are actually deposited in the papillary-like formations of the mucosa where they are known to ulcerate through during life, producing a rare sign which has been called ' Bilharzial haemo-spermatorrhoea.' The epithelium on the top of the vesicular papillary formations where the ova are deposited is very much desquamated.

Sections of the vasa deferentia of this case show the tube with its thick muscular coat embedded in a thick fibrous sheath with heavy deposit of calcified ova and absence of eosinophil cells.

The urinary bladder shows heavy infiltration of calcified terminal spined ova in the mucosa and submucosa, with a good deal of fibrosis. There are also some living ova with cellular reaction around them. The lungs showed some calcified ova in them. The rectum and liver were negative.

CASE No. 4. This is a case of bilharzial fibromyoma of the uterus.

The patient G. E. R., 35 years old, was admitted to the Qasr el Ainy Hospital, complaining of an abdominal tumour which was diagnosed as uterine fibromyoma and operated upon by Professor Naguib Bey Mahfouz, and the uterus was removed by subtotal hysterectomy and a shell of cervix was left behind. She was discharged cured. Her urine was negative for ova but lateral spined ova were found in her faeces.

Naked eye appearance of the tumour after its removal by operation.—The mass is oval and slightly larger than a full term foetal head, hard and fibrous to the touch, and is made up of an intramural fibromyoma originating in the lower part of the posterior wall of the uterus and growing downwards filling Douglas Pouch and also laterally into the right broad ligament. On cutting through the body of the uterus and tumour mass, the former was found lying on and fixed to the upper and anterior aspect of the tumour by a fold of muscular tissue passing from the fundus of the organ on to the outer covering of the tumour. The growth itself is one single fibromyoma which is encapsulated and hard and shows the typical coarse striation and whorled appearance of a fibromyoma.

Microscopically, the mass shows the ordinary picture of a fibro-myoma with lateral spined ova deposited here and there, some living and others dead. Around the living ova is to be seen the usual nodular cellular tissue reaction as well as quite heavy eosinophil cell infiltration both in relation to the ova and away from them in the tumour mass. The presence of these eosinophil cells in the tumour mass undoubtedly altered the histological picture in so far that they seem to have excited the fibrous tissue elements to proliferation with obvious pressure atrophy on the muscular bundles intervening. Thus, where no eosinophil cells are present, the muscular tissue predominates in thick cellular bundles of healthy-looking muscular cells, whereas in the fields with heavy eosinophil cells, the fibroid tissue is found predominating with compressed and atrophied thin muscular bundles. This fibroid tissue infiltrated with eosinophil cells is much more poorly cellular than the fibrous tissue proper of the fibromyoma. This picture again supports the idea that the presence of the eosinophil cells gives a stimulus to fibroid tissue formation, an idea with which my colleague, Dr Sorour, is so impressed that he suggested and is now actually experimenting on the effect of artificially-provoked eosinophilia on the healing of tuberculous and other lesions in experimental animals.

In the light of the great capacity of the schistosome factor to produce actual benign and malignant neoplasms, quite apart from the granulation tissues, the question arises, how far the ova in this fibromyoma may be responsible as causal factors for this neoplasm.

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EXPLANATION OF PLATE XIV

CASE NO. I.

1. Photograph of the papilloma of the sigmoid.   See text.

2. Microphotograph of the above papilloma showing a living ovum with cellular tissue and eosinophiles around it, and four calcified ones with less cellular tissue and more fibrosis around.

3. A low-power microphotograph of a section of the spleen showing two ova with eosinophiles around and no reaction ; the ovum in the centre is partially surrounded by a strip of giant cell.

4. Schistosome nodule in the peritoneum of the gut showing three living ova with whorly fibrosis around them and numerous eosinophiles.   The outer surface of the peritoneum is at the top of the figure.

5. Lymph gland showing the subcapsular sinus with much fibrosis of the capsule and ova, and ova in the parenchyma (Pl. XV).

FIG. 1

FIG. 2

FIG. 3

FIG. 4

EXPLANATION OF PLATE XV

CASE NO. 2.

6. Photograph of nodules in omentum (comp. text p. 566).

7. Low-power microphotograph showing schistosomal follicles in omental tumour.

8. Worm in the subomental tissue and calcified ova with thickened fibrous omentum.

FIG. 5

FIG. 6

FIG. 7

FIG. 8

EXPLANATION OF PLATE XVI

CASE No. 3.

9. Photograph of seminal vesicles fused together, prostate and vasa (comp. text p. 567).

10.—Seminal vesicle showing bundles of fibrous tissue with calcified ova amongst them ; also shows lumen of the vesicle.

CASE No. 4.

11.—Photograph of the fibromyoma of the uterus (comp. text p. 568).

12.—Low-power microphotograph of a section of the fibromyoma showing calcified ova and no reaction around them.

Fig. 9
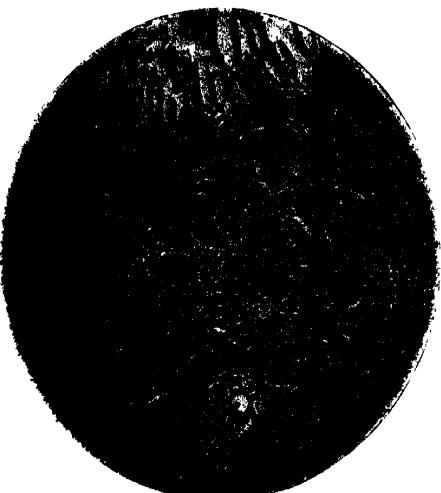
Fig. 10

Fig. 11

Fig. 12

THE EARLY STAGES OF THE JIGGER, *TUNGA PENETRANS*

BY

E. P. HICKS

(From the Sir Alfred Lewis Jones Research Laboratory, Freetown, Sierra Leone)

An insect which is of so peculiar a nature as the jigger, and which refuses to be disregarded, must have figured largely in the early travellers' tales of Central and South America. As long ago as 1551 it was noticed by Oviedo, and since that time has been described by numerous authors under many popular and scientific names, such as the Chigger or Chigoe, Pique, Nigua, and Bicho de pé, and by scientists as *Pulex, Dermatophilus, Sarcopsylla* and *Rhynchoprion penetrans*. It should be noted that the name ' jigger ' is also applied in North America to a mite. A list of authors is given by Jordan and Rothschild (1906).

As might be expected, it was only a question of time before improved communications allowed it to spread to other parts of the world. From Brazil it appears to have reached West Africa in 1872 or 1873, travelling in the British ship ' Thomas Mitchel,' Bahia to Ambriz in ballast (Blanchard, 1889, Taschenberg, 1880). The ballast consisted of sand, which was unloaded on shore, contrary to the regulations, which prescribed that it should be discharged into the sea. From Ambriz it spread north and south along the coast, and also so quickly inland that in 1875 it was found about seventy miles from the coast. With the help of Stanley's expedition and Arab caravans, it has travelled across the continent to Tanganyika, Madagascar (Blanchard, 1899), the Sudan (Wellcome Research Laboratory, 1906), the Transvaal (Spencer, 1912), Seychelles (Addison 1915), and Kenya (Anderson, 1924). It has not, however, established itself in India (Turkhud, 1928), nor in Europe, though it has been introduced on several occasions. It is worth observing that, though it was formerly prevalent in Bermuda, it has since disappeared

(Balfour, 1928). It is now a widespread and important pest, and even if it does not compete in destructive action with *Xenopsylla cheopis*, it is yet so unpleasant in its effects that Sir Harry Johnston (1897) said that it 'is supposed to be the origin of the sailor's oath— " Well, I'm jiggered ".'

The larvae of several species of flea have now been described, of which a useful list of references is given by Sikes (1930). The larva of *Tunga penetrans* was first described by Bonnet (1867), a French naval surgeon serving in French Guiana. His description is in most respects very valuable, but undoubtedly contains some errors. There is also a brief account of the larva by Newstead, Dutton and Todd (1907). It was thought, therefore, that a fresh description of the early stages of this flea would be of interest.

Material and Methods. The material described in this paper was obtained by extracting jiggers from the feet of African children living in Freetown, Sierra Leone. Each flea was placed in a separate petri dish, and many laid eggs immediately after extraction. Some of the larvae and pupae developing in each dish were killed for examination, while others were bred out to the adult stage in order that the species might be determined with accuracy in each case. To determine the number of instars, a series of small glass cells were prepared, in each of which three eggs were placed. The larvae which hatched from these eggs were reared under the same conditions as to food and moisture as those in the petri dishes, but as they were segregated, it was possible to observe them closely, and collect the cast skins for examination. The bottom of each petri dish was covered with a thin layer of road dust (laterite) which had been heated to destroy other organisms. The larvae were unable to exist on bare glass or on filter paper.

Bonnet stated that his larvae did not survive unless fed on the dead body of the mother. It is true that they will eagerly nibble at the corpse, but they will thrive equally well on other food ; and, indeed, if one considers that the eggs are broadcast on the ground, it will be seen that the larvae can rarely be in a position to nourish themselves on the body of the mother flea, a point which Bonnet realised. In my cultures a number of foodstuffs were tried. Threads soaked in blood and dried, or blood dried and powdered were used at first, but it was found that the larvae developed more

rapidly and with fewer casualties on a more mixed diet. It was also found essential that the food should not be too moist. Though larvae would eagerly attack such liquid food as palm oil, they found themselves unable to emerge from it after their meal, and soon died. The following method was finally evolved. At the time of this work there were always available in the laboratory a number of larvae of *Cordylobia anthropophaga*. The viscera of these were placed on a glass slide in the incubator at 37° C. for about two hours. They were then scraped off in flakes and placed in corked test tubes, in which form they could be kept for several weeks. When required for use, the dried viscera, with some dried blood, were placed in a test tube, to the cork of which was fastened a pad of moist cotton-wool. After one or two days the surfaces of the particles of food became damp and sticky. A little dry road dust was thrown into the tube and the mixture shaken vigorously. The dust adhered to the food and protected the larvae from its surface which must necessarily become adhesive in a damp atmosphere. The temperature of the laboratory fluctuated little. The daily minimum for the period of the work varied between 76° F. and 82° F., the daily maximum between 78·5° F. and 86° F. The relative humidity was more variable, in one case rising to 96 per cent., and in another falling to 54 per cent. As it was found that the larvae died when the air was dry, they were kept in a damp atmosphere maintained by fixing a pad of filter paper to the inside of the lid of the petri dish with adhesive plaster, and keeping it constantly moistened. Therefore, it may be said that the breeding was carried out at a temperature of about 80° C. and in a nearly saturated atmosphere.

For the determination of the structure, the larvae were cleared and mounted in creosote or Berlese's fluid ; or fixed in Bles's fluid and cleared and mounted in carbol-alcohol. To display the chitinous structures of the head, it was teased up in a small quantity of Berlese's fluid. The structures were more easily separated when the larva had been soaked in cold 10 per cent. potash for one or two hours. The dissection of the viscera was carried out on a coverslip by the usual methods. The head was removed from the body, drawing with it the viscera ; the preparation was washed in normal saline and the organs separated from each other by cutting the tracheal connections ; the tissues were fixed to the coverslip by cautiously

flooding it with fixative (Bles or Bouin). It was then stained with Mayer's acid haemalum.

Structure of the Early Stages. The eggs (fig. 1, A) are oval, resembling in shape those of other fleas. They measure 0·48 to 0·61 mm. in length, by 0·28 to 0·33 mm. in breadth. The colour is pearly-white.
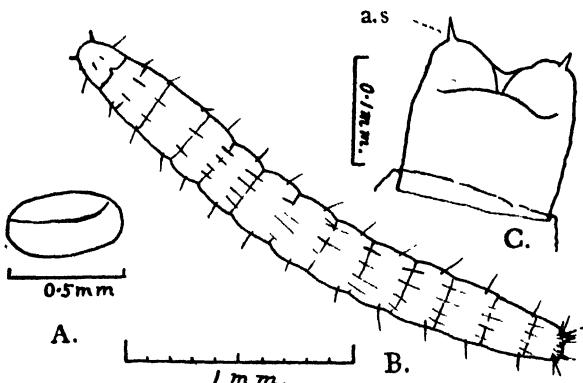
Fig. 1. A.—Egg; B.—First instar larva; C.—Terminal segment of first instar larva; *a.s.*—anal strut. Drawn by camera lucida.

Larval Stages. Tunga penetrans has only two larval instars, in contrast to the larvae of other fleas which have been described. Oudemans (1913), however, observed only two instars in *Histrichopsylla talpae* and *Spalacopsylla bisbidentatus*. To confirm the absence of a third instar in *T. penetrans*, larvae were segregated in cells, as described above and examined daily. Cast skins were obtained from all larvae, and all the skins showed an egg-breaker. A careful search of the dust in which the larvae were living revealed no skins without an egg-breaker. Such skins could only be found inside a cocoon after pupation. Besides this, a large number of skins obtained from the ordinary petri dish cultures were examined. All of these showed an egg-breaker, except, of course, those from the interior of a cocoon.

The First Instar Larva (fig. 1, B) emerges by inserting the egg-breaker into the shell at one pole, and drawing it down the length of the shell, cutting a longitudinal slit with slightly curved ends. The length of the first instar larva is 1·4 to 2·5 mm. and the breadth

is 0·17 to 0·3 mm. The measurement of the killed larva, however, is not a very exact proceeding, as the dimensions depend to some extent on the degree of contraction of the body. It consists of a head, three thoracic and ten abdominal segments, the last of which is produced into two lobes, each of which bears a chitinous anal strut. The strut is in the form of an isosceles triangle (fig. 1, c). The surface of the thoracic and abdominal segments is rough, with the exception of a smooth area on the dorsum of each segment. In the first to the seventh abdominal segments there is a shallow longitudinal groove on each side, which separates off a narrow lateral flange. Each segment bears a number of hairs arranged in a single transverse row, rather nearer the posterior than the anterior border. The thoracic segments each bear eight hairs; the first eight abdominal segments ten; the ninth abdominal segment has twelve hairs of equal length, and the last segment about seventy-five of unequal length. The number of hairs on each abdominal segment shows some slight variation.

The superficial appearance of the dorsal and lateral aspects of the head is shown in fig. 2, A, C, D. On the dorsal aspect there is an antero-posterior suture on each side of the middle line, the epicranial suture. The egg-breaker (fig. 2, A and C and fig. 3, A) is triangular with the sharp apex directed upwards. The hairs are sufficiently shown in the figures. From the mouth opening protrude the labrum and maxillae. On the anterior part of the ventral surface, on each side, are two backwardly directed hooks. Such hooks are not mentioned in the descriptions of other flea larvae to which I have had access.

The antenna (fig. 3, A) is situated on the antero-lateral aspect of the head. It consists of a cylindrical shaft, which is slightly narrower distally. At the apex is a stout bristle surrounded by four minute hairs. The base of the antenna is mounted on a raised area, ' like an inverted saucer ' (Bacot and Ridewood, 1914), which is bounded above by a thickening of the cuticle and bears three large and three small blunt papillae. In one case there were four large papillae. In many specimens, however, it is difficult to see the full number of small papillae. Near the apex of the antenna is a cavity, which carries two fine hairs internally, and opens to the surface on the posterior aspect of the antenna. This is presumably a sense

organ. Some authors describe two or three segments in the antenna of the larval flea. The number seems to depend on the decision whether or not the 'inverted saucer' and the terminal bristle are to be counted as segments. No joint was perceived in the shaft of the antenna.

The mouth parts consist of the labrum, mandibles, maxillae and. labium.
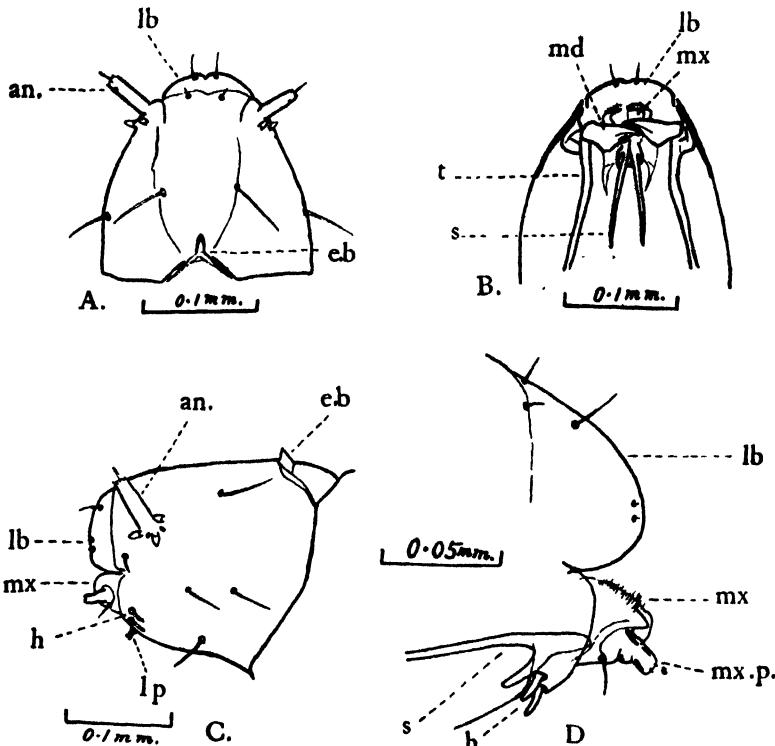
Fig. 2. First instar larva. A.—Superficial aspect of dorsum of head; B.—Deep structures of head; C.—Lateral aspect of head; D.—Lateral aspect of mouth; *an.*—antenna; *e.b.*—egg-breaker; *b.*—hook; *lb.*—labrum; *l.p.*—labial palp; *md.*—mandible; *mx.*—maxilla; *mx.p.*—maxillary palp; *s.*—stipes; *t.*—tentorium. Drawn by camera lucida.

The dorsal surface of the labrum is smooth and carries a few hairs ; the ventral surface has a median groove and is covered with fine hairs.

Examination of the internal structures (fig. 2, B) shows clearly the mandibles, maxillae and two pairs of long narrow chitinous rods lying in an antero-posterior direction.

The mandibles when at rest lie within the head capsule (fig. 2, B), but when in action are rapidly protruded and retracted, and can be seen shovelling the food into the pharynx. The mandible (fig. 3, C) is roughly triangular in shape, the base bearing teeth, the apex being rounded into an articular surface. The angle between the anterior margin and the base is formed by a large blunt tooth, behind which is a second similar but smaller tooth, and a third, which is again smaller. These are followed by a row of three slender sharp teeth.
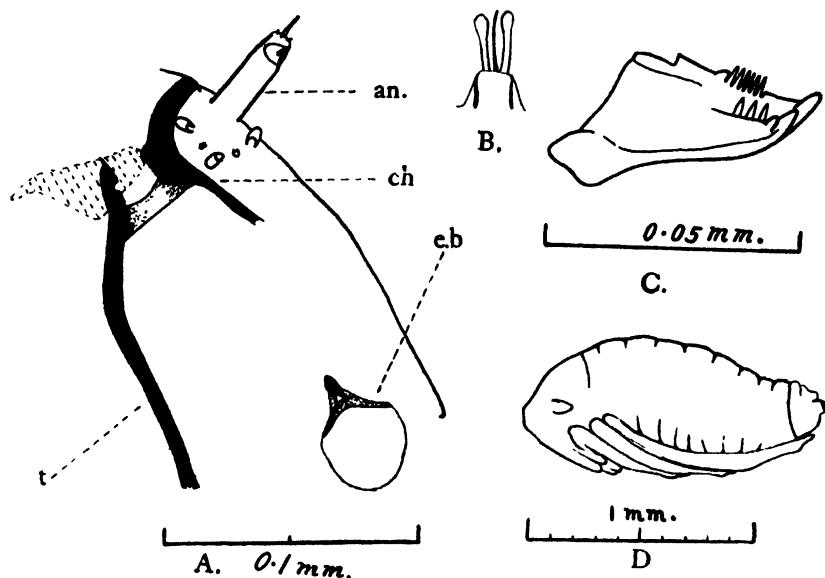
Fig. 3. A.—Articulation of mandible, antenna, tentorium, and egg-breaker, first instar; B.—Labial palp; C.—Mandible; D.—Pupa; *an.*—antenna; *cb.*—chitinous thickening bounding antennal area; *e.b.*—egg-breaker; *t.*—tentorium. All drawn by camera lucida, except B.

Besides these, the margin of the base is indented to form a second row, which has six needle-like teeth, lying parallel to those described above. It shows at the posterior end a notch for muscular attachment. The number of teeth, however, shows some slight variation and may be in each row six or seven, or, less often five. The articular surface of the mandible is applied to a tubercle which may be seen lying internal to the antennal area.

The maxillae (fig. 2, D) lie ventrally to the labrum and mandibles. They are conical in shape. The inner surface of each is covered with

fine hairs. On the lower and outer surface of the maxilla is a recess for the palp; this recess is bounded posteriorly by a thin plate of chitin, bearing a hair. The palp consists of two segments and bears three fine hairs at the apex. At the posterior end of the maxilla is a rod of chitin which from its pointed posterior end runs forward into the base of the maxilla, bearing a recurved arm on its lower and outer aspects. This is the stipes.

Of the two pairs of chitinous rods mentioned above, the inner and shorter pair are the stipes. The outer pair are longer and stouter. They are rod-shaped sclerites representing the tentorium, and extend to the posterior extremity of the head capsule. If one is traced from its posterior end, it is seen to run forward, pursuing a slightly curved course to end in a sharp point just lateral to the hooks on the ventral surface of the head. Behind the point the sclerite has a recess on its lateral margin, and on the posterior edge of the recess there is a tuberosity. Immediately posterior to the tuberosity a thin plate of chitin runs outwards and upwards, and is continuous with the tubercle forming the articular surface for the mandible. This last portion of the sclerite is connected with the chitinous thickening which bounds the antennal area.

On the ventral aspect of the head, the labial palps can be seen posterior to the maxillae, projecting between the two pairs of ventral hooks. Each palp (fig. 3, B) carries three processes, of which two are club-shaped and one needle-shaped.

Bonnet's account of the labial palp has given rise to some confusion. He wrote ' De chaque côté de la lèvre (supérieure) on remarque une éminence arrondie, au sommet de laquelle on voit saillir un prolongement assez court de forme cylindrique et supportant à son extrémité libre trois petites soies. Ce prolongement représente le palpe labial ; il peut, à la volonté de la larve, rentrer à l'intérieur de l'éminence qui lui sert de base.' The ' palpe labial ' is undoubtedly the maxillary palp, as Taschenberg (1880) pointed out. The true labial palps lie on the ventral aspect of the head in the middle line, with a pair of hooks lying laterally to them on each side. Bonnet missed the labial palps, but saw the hooks—' Quatre crochets servant à la progression de la larve.' Künckel (1873), working with the larvae of *Ct. felis* and *C. fasciatus* described a pair of labial palps but saw no hooks, and, therefore, wrote that the ' quatre crochets '

of Bonnet were the true labial palps. In reality there are in the larva of *T. penetrans* both hooks and labial palps.

The gut and salivary glands are identical in morphology with those of the second instar.

When the time for ecdysis has arrived, the larva flexes sharply the head and first two segments, and after a struggle succeeds in splitting the first instar cuticle on the dorsal aspect and emerges. Sometimes the larva fails to free itself from the last few segments of the cast skin and dies. In fact there were usually a number of deaths at the time of the first ecdysis.

The Second Instar Larva measures 2·0 to 3·1 mm. in length by 0·22 to 0·4 mm. in breadth. Its structure is the same as that of the first instar, with a few exceptions. There is, of course, no egg-breaker. The anal struts are much smaller than those of the first instar, and in many specimens could not be distinguished.
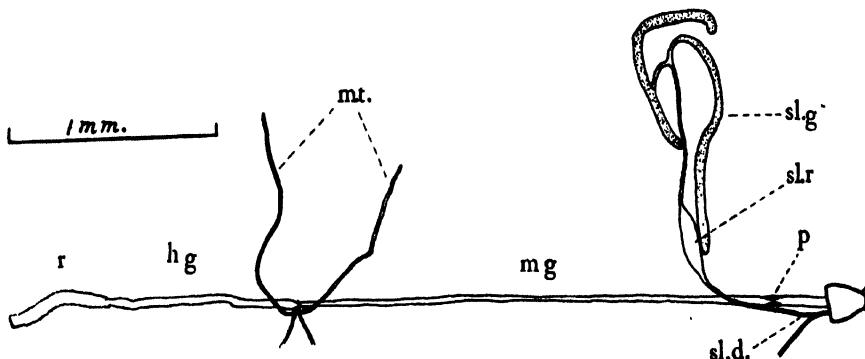
Fig. 4. Salivary glands of one side and gut of second instar larva. *hg.*—hind gut; *mg.*—mid gut; *m.t.*—Malpighian tube; *p.*—proventriculus; *r.*—rectum; *sl.d.*—salivary duct; *sl.g.*—salivary gland; *sl.r.*—salivary reservoir. Drawn by camera lucida.

The Viscera (fig. 4) were studied by dissection in the first instar larva (in most cases unfed), or in the second instar larva which had emptied its gut prior to pupation.

The length of the gut in the second instar, measures from the posterior border of the head to the anus, about four millimetres. The proventriculus, shortly behind the head, is formed by a slight thickening of the circular muscle. The midgut is rather more than half the total length of the gut. It is a narrow thin-walled tube,

the cells of which appear uniform throughout. It is separated from the hindgut by a valve, which is formed by a thickening of the circular muscle coat. The hind gut has walls thicker than those of the midgut. It shows no specialised group of cells. Towards the posterior end of the body, on its course to the anus, the gut is surrounded by a thick layer of circular muscle, which constitutes the rectum. There are four Malpighian tubes taking origin from the gut at the level of the pylorus.

Each of the two salivary glands is composed of three lobes, with ducts and a reservoir. The lobes are unequal in length. The longest is distinct from the other two, and is attached by its tip to the wall of the reservoir. The other two lobes arise together, inasmuch as the secreting cells are common to both ; but after a very short course the lobes separate. The shorter of the two is variable in length ; sometimes it consists of only two or three cells ; in one specimen it appeared to be absent. The lobes are composed of a double layer of spheroidal cells bounding the lumen ; the cytoplasm is finely granular, and the nucleus is round or oval. At the proximal end the cells are smaller and are cubical, forming a duct which unites with a similar duct from the other part of the gland to form the salivary duct. The walls of the salivary duct are formed by a single layer of flattened cells. After a short course it is dilated to form a thin-walled reservoir, from which it is continued to the posterior border of the head, to join the duct from the other side of the body, thus forming a common salivary duct. The common duct and that portion of the salivary duct which lies proximal to the reservoir are fine tubes with annular markings ; they have the appearance of being formed of a number of closely applied rings, reminiscent of the human trachea. There is a long narrow fat body on each side.

The Pupa. When the larva is ready to pupate it empties its gut and encloses itself in a thin-walled cocoon, to the outside of which particles of dust adhere. If the cocoon is opened, the larva is found to be sharply folded on itself, so that the terminal segment lies close to the head. The posterior part of the body becomes slightly broader and more opaque than the anterior part. It then casts its skin and becomes a pupa.

The pupa (fig. 3, D), resembles in general that of other fleas, but

shows plainly the characteristic shape of the head and thorax of *Tunga penetrans*. The pupal case is a delicate cuticle, which is closely applied to the pupa, and continued in tubular form over the appendages. At first the colour is white, but later changes to the reddish brown colour of the adult.

Distinguishing features of the larva of T. penetrans.

The larva differs from the larvae of other *Pulicidae* which have been described in the following particulars :—

The existence of only two larval instars.

The form of the egg-breaker.

The form and number of teeth on the mandible.

The form of the maxillary palp.

The appendages of the labial palp.

The possession of four hooks on the ventral surface of the head.

The arrangement of hairs on the abdomen in a single transverse row.

The form of the anal struts.

Duration of Stages of development.

The larva emerges on the 3rd to 4th day after the egg is laid.

The first ecdysis takes place on the 5th to 8th day after the egg is laid.

The larva prepares for pupation on the 6th to 17th day after the egg is laid.

The larva pupates on the 10th to 18th day after the egg is laid.

The imago emerges after the 17th day after the egg is laid.

I am much indebted to Professor W. S. Patton for advice and assistance in the preparation of this paper.

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ON CERTAIN DISTINGUISHING CHARACTERS OBSERVED IN *ANOPHELES FUNESTUS* GILES

BY

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In his ' Provisional List and Reference Catalogue of the Anophelini,' Christophers (1924), restricted the definition of *A. funestus* Giles, to include only the African form of the group, the Oriental *A. minimus*, *A. aconitus* and *A. listoni* being regarded as distinct species. With regard to the last-named species, however, it was stated that the question still arises ' whether this is a variety only of *A. funestus* or a distinct species.' In their synoptic table of the Indian Anophelines, Christophers, Sinton and Covell (1927) still regard the three oriental forms as distinct species. The identity of some of these Oriental species has, however, recently been called in question by Manalang (1930) who, referring back to a paper by Strickland (1924), published before the appearance of Christophers' Memoir referred to above, gives his reasons for regarding the form of *A. minimus* found in the Philippines as synonymous with *A. funestus* Giles. A new variety of *A. aconitus* from the Philippines, is described in the same paper.

In the paper referred to, Manalang calls attention to certain larval characters which he finds useful in distinguishing the Philippine *minimus* (' *funestus* ') from another Philippine form of the group, the newly described *A. aconitus* var. *filipinæ* Manalang. A comparison between Manalang's description of the larvae of Philippine ' *funestus* ' and those of a series of bred *funestus* from the type locality, Freetown, Sierra Leone, has led to the discovery of a well-marked difference which may perhaps be of value in further establishing the distinctness of *A. funestus* from *A. minimus* and possibly also from *A. listoni* and *A. aconitus*. In addition to this, a character common to the males of African *funestus* has also been investigated and shows what appears to be a constant difference from *A. minimus*, *A. listoni* and *A. aconitus*. The larval material on which the following observations

are based, comprises a few larvae from which I bred out adults of *funestus* in Freetown, in 1925, four larvae collected in Freetown by Professor Blacklock and the writer in the same year, and four larvae collected near Stanleyville, Belgian Congo, and kindly presented by Dr. J. Schwetz ; some of this latter series were bred out, giving rise to *funestus* adults. Oriental larvae examined were a series of *A. minimus* var. *varuna* identified by Dr. M. O. T. Iyengar, and one *A. listoni* and two *aconitus* larvae from Ceylon, identified by Mr. H. F. Carter ; the larvae were presented by these entomologists to Professor Patton, to whom I am indebted for permission to examine them.
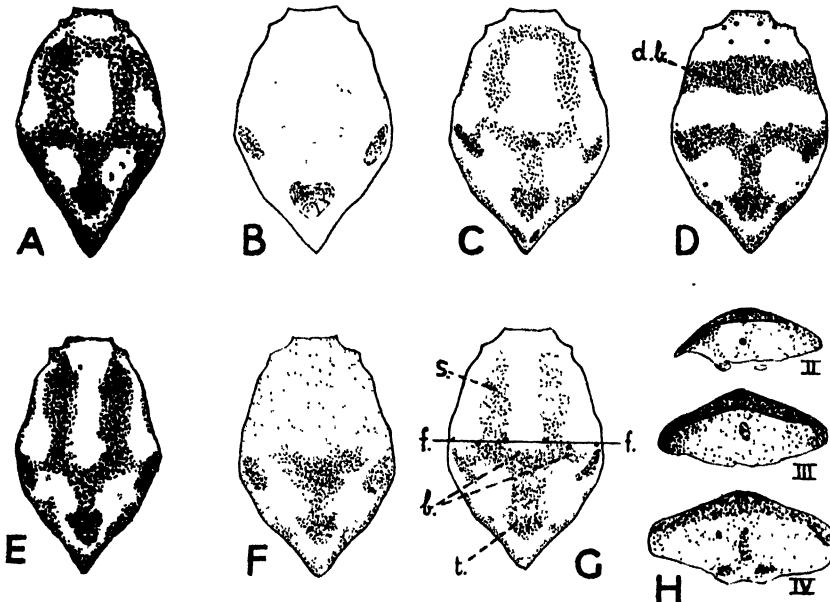
Fig. 1. A to E.—Fronto-clypeus of fourth stage larva, to show pattern ; A.—*Anopheles aconitus* var. *filipinae* ; B, C, F and G.—*A. minimus* var. *varuna* ; D.—*A. funestus* ; E.—*A. minimus* ('*funestus*'), Philippine form ; H.—Tergal plates of second to fourth abdominal segments of larva of *A. funestus* (a-typical). A and E copied from photographs in Manalang (1930), other figs. original. The markings in D should be darker than shewn.

The larval character described by Manalang, which I find to be distinct in African *funestus* larvae, is the fronto-clypeal pattern. The great value of this character in separating larvae of *A. sergenti* from those of *superpictus* was pointed out by Buxton (1923).

The differences observed in this character are described with reference to the diagram (fig. 1, G), which represents the pattern seen

in some specimens of *A. minimus* var. *varuna* Iyengar. The transverse line, *f.f.*, drawn across the front at the level of the mid frontal bristles, divides the pattern into a proximal portion which, as Buxton has pointed out, is chiefly a broad transverse ' butterfly-shaped band.' This band is lettered *b.* in the diagram. In many specimens it is connected with the basal triangular patch *t.* Beyond the level of the line *f.f.*, the pattern consists of two longitudinal stripes *s.*, which are usually continuous with the transverse band *b.* Fig. 1, B, C, F and G, illustrate some variations seen in eight fourth stage larvae of *A. minimus* var. *varuna* examined. In none of the specimens examined was the pattern dark, and in several it was very light. The pattern shown in fig. 1, G, was seen in three out of the eight larvae studied. In three others there was a reduction in the dark areas, the main centres of chitinisation being more or less isolated from each other, as in fig. 1, B ; it is possible, however, that these were recently moulted fourth stage larvae. Fig. 1, C, illustrates the condition seen in one of the larvae, the main difference from that shown in fig. 1, G, being that the two longitudinal stripes are connected across anteriorly to form an ' O ' shaped pattern in front. It is interesting to note that this pattern agrees in this respect with that of Manalang's *aconitus* var. *filipinœ*, while that illustrated in fig. 1, G, resembles that of the Philippine *minimus* ('*funestus* '). Figs. 1, A. and E, are copied from Manalang's photographs. In one larva of *minimus* var. *varuna*, in which the imaginal buds appeared to be in an advanced state of development, practically the whole of the anterior half of the fronto-clypeus was chitinised, as shown in fig. 1, F. Thus it is seen that a considerable amount of variation exists in *A. minimus* var. *varuna* with regard to this character.

In the larva of *listoni* and two *aconitus* larvae I have examined, the pattern could not be made out very clearly on the anterior quarter, but in both cases it was evident that longitudinal marks continuous with the proximal transverse band were present, the general plan being that shown in figs. 1, C. and G, but in all three the chitinisation was much heavier than in the var. *varuna* larvae, and the markings appeared rather more extensive than in fig. 1, G.

In all our African larvae of *A. funestus* in which this character can be clearly seen, the pattern is quite different from that seen in

any of the above described variations of *minimus* var, *varuna*, and from that of the larvae of *listoni* and *aconitus* I have seen, and also from those illustrated by Manalang for the Philippine forms of the group. A typical pattern is that illustrated in fig. I, D, which was drawn from a larva collected in Freetown, West Africa. It will be seen that the proximal part of the pattern shows little difference from that of the Oriental forms ; it may show slight differences, as shown in the illustration on page 12 of my memoir (1927). On the distal half, however, the difference is striking. Instead of two longitudinal stripes there is a broad transverse band, *d.b.*, extending across the whole width of the front and separated from the proximal band by a space about equal to the width of the distal dark band. The occurrence of this separate transverse dark band across the distal third of the fronto-clypeus is a constant feature in the seven larvae, including specimens from Sierra Leone and Stanleyville, in which the pattern can be clearly made out.

Manalang has also found that the presence or absence of chitinous ' islets ' in the membranous part of the abdominal segments dorsally is a character which distinguishes the two Philippine forms. It is interesting, therefore, to note that in twelve African *funestus* larvae examined, ' islets ' are usually absent, but one larval pelt shows the condition illustrated in fig. I, H. In some other larvae, ' islets ' were represented on most of the segments by thickenings of the tergal plate, as in the fourth segment of the larva illustrated.

Adult character. In their 'Synoptic Table for the Identification of the Anopheline Mosquitos of India,' Christophers, Sinton and Covell (1927), illustrate the male palps of *A. listoni* and *A. minimus ;* *A. aconitus* is said to resemble *minimus* in this respect. In the illustrations given it is seen that in typical specimens the club shows a pale spot or band (*listoni*) or broad pale area (*minimus* and *aconitus*) at the base. From other descriptions seen and examination of the male palps of specimens of *minimus* and *listoni* in the collection of the Liverpool School of Tropical Medicine, including examples determined by Colonel Christophers, it seems certain that white scales in the form of a well-defined band or area or sometimes a small spot are practically always present at the base of the palps in these Oriental species. In one specimen from Hong Kong determined by me as *minimus*, there was only a very small pale spot in this position.

In specimens of *funestus* from Africa that I have examined, however, the base of the club of the male palp was invariably without any pale scales. The amount of white on the outer half of the club shows a great deal of variation. Sometimes it is reduced to one or two very small spots and in other exceptional cases the whole or almost the whole of the outer half of the club is pale scaled. Fig. 2, C, which was drawn from a specimen bred from a larva taken in Freetown, Sierra Leone, represents a typical condition met with in African *funestus*. In addition to specimens from Freetown and
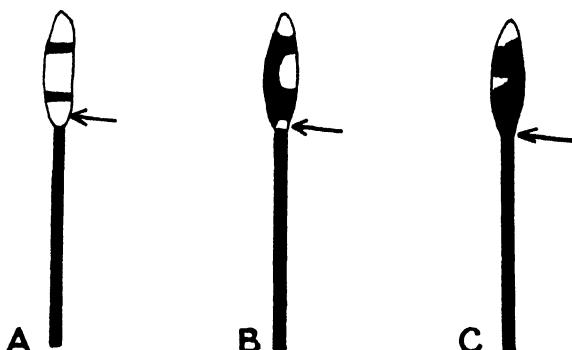
FIG. 2. Male palps, diagrammatic. A.—*A. minimus*; B.—*A. listoni*; C.—*A. funestus*. A and B after Christophers, Sinton and Covell (1927), C original.

elsewhere in Sierra Leone, I have examined the male palps in *funestus* from the Gold Coast, Nigeria, Belgian Congo, Kenya, Pemba, Nyasaland and Mauritius; two males of *funestus* var. *fuscivenosus* Leeson, kindly presented by Mr. Leeson, also showed the dark base of the palps. This character also seems to be a good distinction between *A. funestus* and small specimens of *A. transvaalensis*.

It has frequently been pointed out that characters formerly used to separate *funestus* from *minimus* and *listoni* are subject to a certain amount of variation. Even the width of the subapical pale band on the female palp of *A. minimus* is sometimes seen to approach a condition frequently seen in *funestus*. This is so in a specimen I have determined as *minimus* from Hong Kong, the subapical pale band being considerably less than the dark band. So far as the material I have examined goes, however, the characters described here appear to show definite discontinuity between *A. funestus*, as

restricted by Christophers (1924) on the one hand, and *A. listoni*, *A. minimus* and *A. aconitus*, on the other. The number of larvae examined is too small to form the basis of definite conclusions, but it is suggested that investigation of these characters in many more specimens of all these species may help to establish the distinctness of *funestus* from the Oriental species of the group.

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PLASMODIUM OVALE

BY

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AND

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Stephens (1922), in a paper entitled ' A New Malaria Parasite of Man,' gave a description of a malaria parasite found in the blood of a patient who was infected in East Africa. This parasite, to which he gave the name *Plasmodium ovale*, resembled *P. malariae*, but the infected red cells were normal in size or only slightly enlarged, frequently oval and showed Schüffner's dots. In this paper, Stephens stated that the parasite appeared to resemble that found by Ahmed Emin (1914), in six pilgrims at Camaran, in the Red Sea, and described by him as *P. vivax* var. *minuta*, but as he was unable to procure Emin's specimens for examination, he could not reach a definite decision in the matter.

Five years later, Stephens and Owen (1927) recorded the discovery of another instance of infection with *P. ovale* in a man who had contracted malaria in Nigeria. Their description of this parasite agrees in all respects with Stephens' original description. In their conclusion they write, ' We are unable to identify *P. ovale* with *P. vivax* var. *minuta* Emin. *P. ovale* is a quartan-like parasite. Infected red cells have a characteristic shape and are stippled, decolourised and slightly enlarged. What relationship, if any, this parasite has to the quartan parasite remains to be seen.'

In July of the present year, we admitted to hospital a patient from Nigeria, suffering from chronic arthritis. After he had been in hospital a few days he developed an attack of malaria, and examination of his blood showed an infection with a parasite indistinguishable from that described by Stephens as *P. ovale*. With a view to studying this parasite more closely and with the object of ascertaining whether its peculiar characters were constant, we

passed it through a series of five general paralytics or tabetics, by direct blood inoculation. The passages are shown in the following genealogical table.

```
S.——————— nat. infection Nigeria
           |
  ┌────────┴────────────────────┐
McC.——————(4 July)      B.—————— (4 July)
                                |
                        Gl.—————— (26 July)
                                |
                        K.—————— (15 Aug.)
                                |
                        Gr.—————— (23 Aug.)
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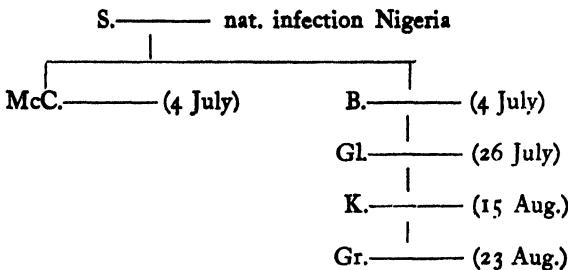
As the result of this study we are able to add little to what Stephens wrote in his original paper. The parasite itself appears to be indistinguishable morphologically from *P. malariae*. It differs, however, in that its cycle of schizogony is completed in forty-eight hours, and temperature charts of infected cases are of the tertian type. This is particularly well shown by the chart of experimentally infected Case K.

The characteristic appearance of the infected red cells, to which Stephen's attention was originally drawn, was clearly seen in the naturally infected case and in all five subinoculated cases. The infected red cells are moderately enlarged—occasionally enormously so ; they are pale, fragile, frequently oval, usually with irregular ragged outline, and heavily stippled.

Whether these characters, viz., the quartan-like morphology, the forty-eight hour schizogony cycle, and the peculiar changes in the infected red cells, are sufficient to justify the erection of a new species, we do not know ; but that these characters are constant and that they serve to enable the parasite to be immediately recognised there seems to be no reason to doubt.

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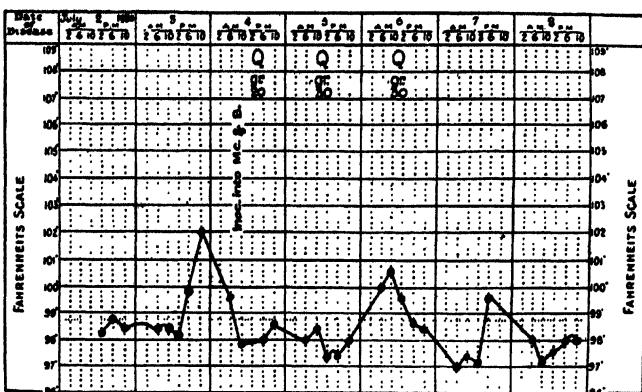
FIG. 1. Case S. Natural Infection.
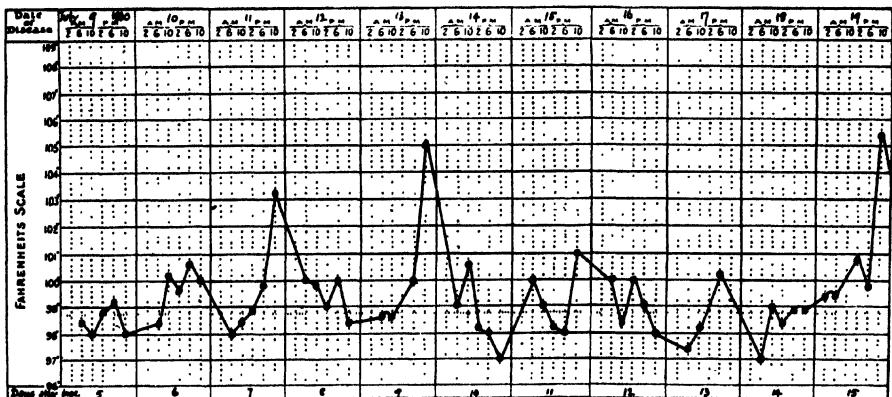
FIG. 2. Case McC. Induced Infection.
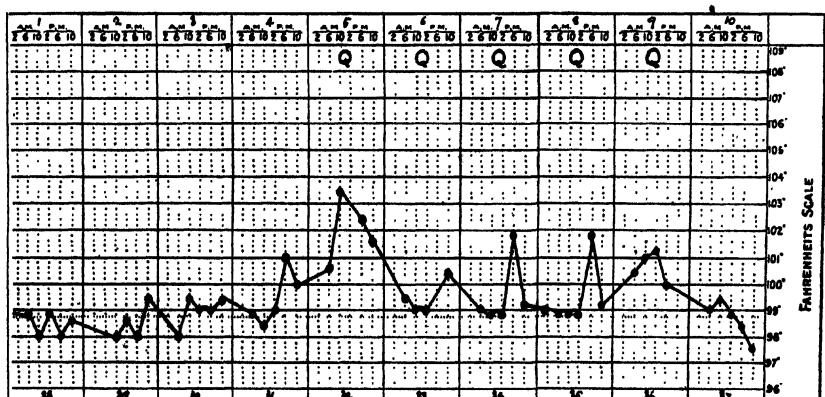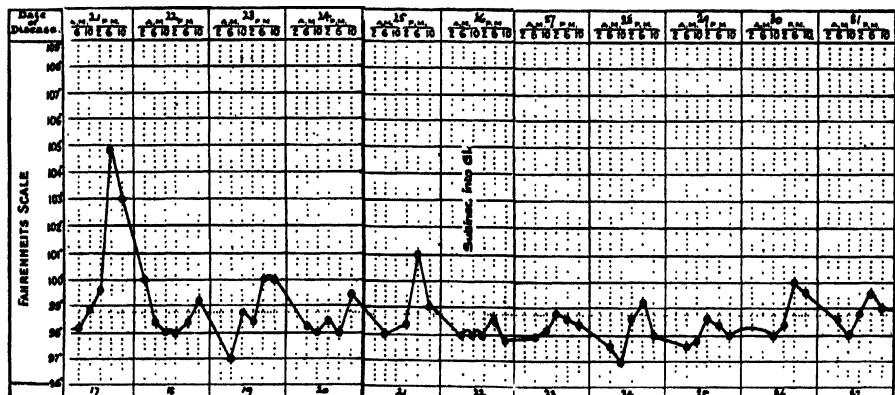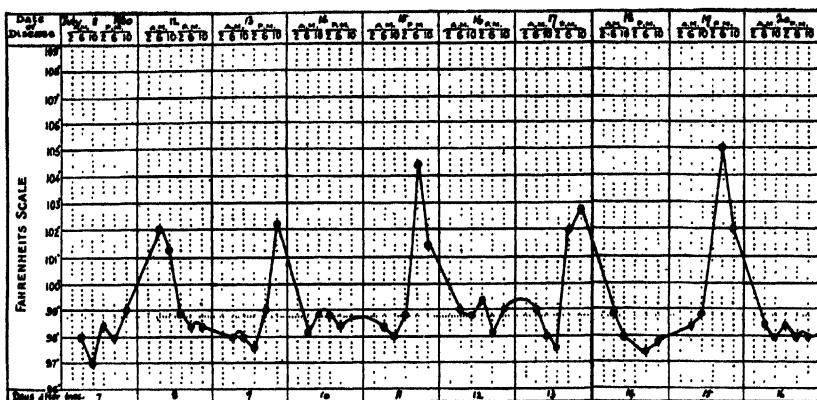
FIG. 3.   Case B.   Induced Infection.

FIG. 4. Case G1. Induced Infection.
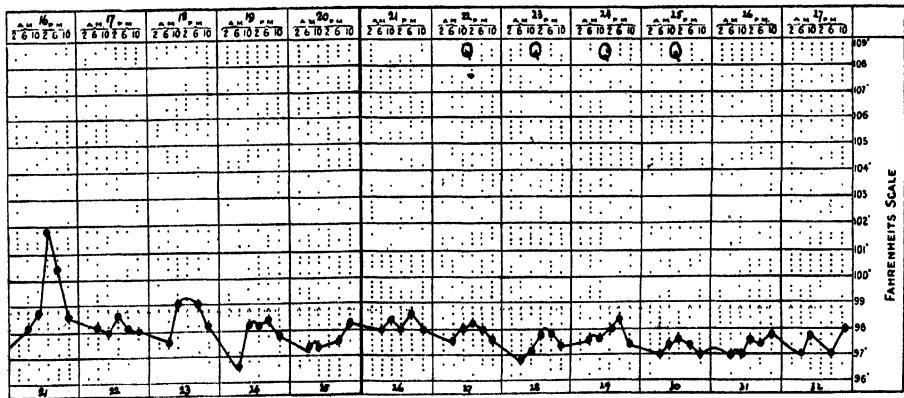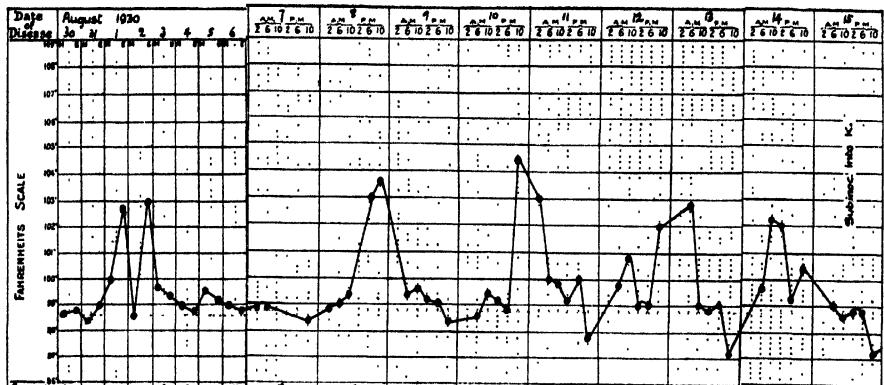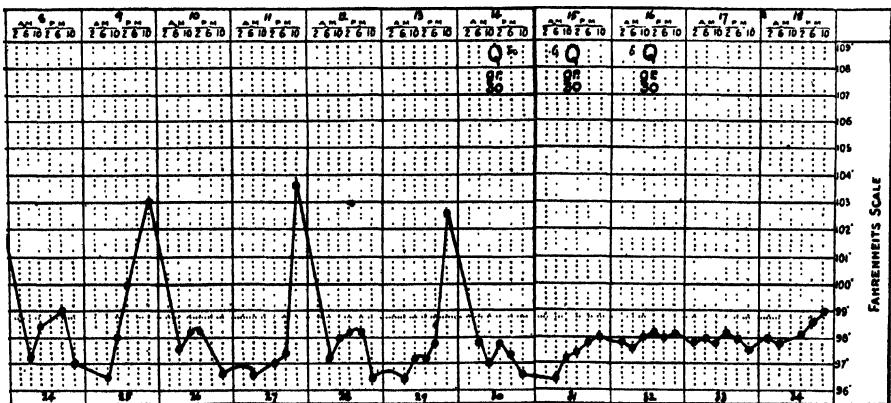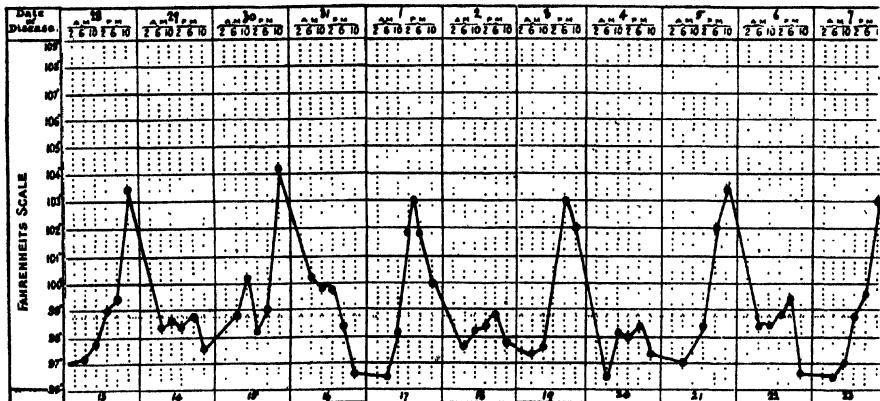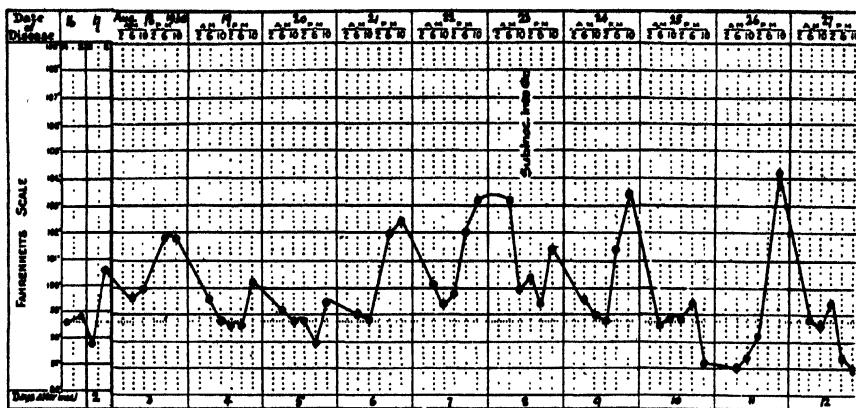
FIG. 5. Case K. Induced Infection.
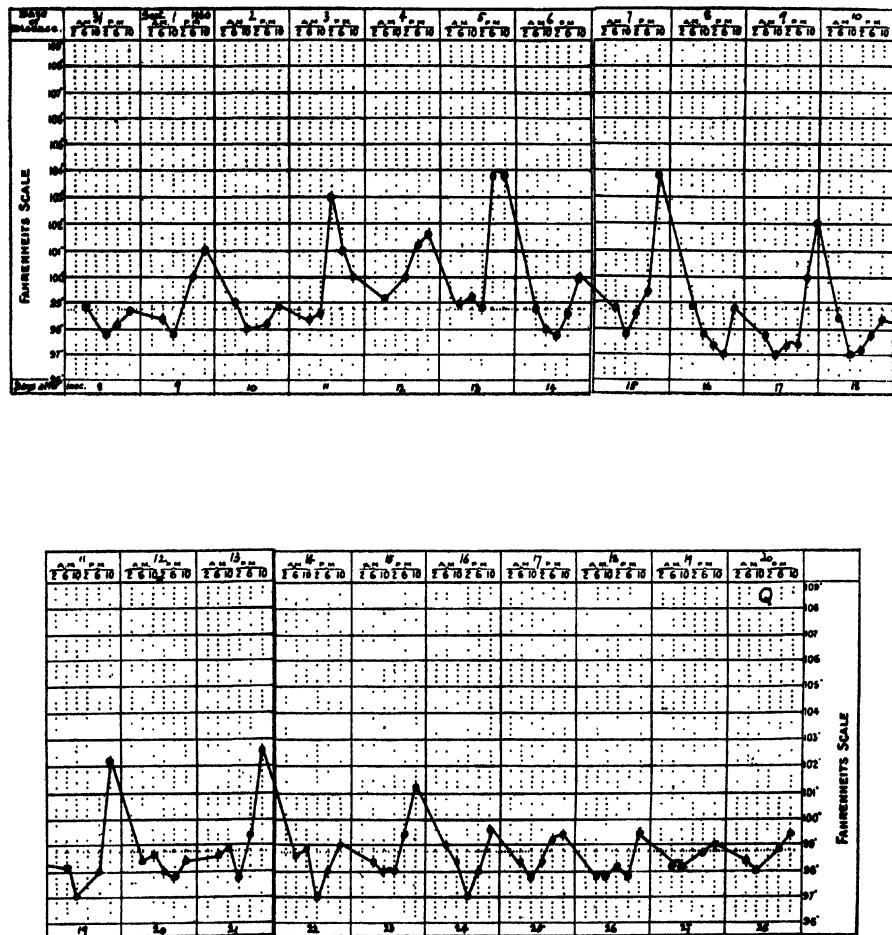
FIG. 6.   Case Gr.   Induced Infection.

MISCELLANEA

A CASE OF KALA-AZAR FROM WEST AFRICA

Cases of Kala-Azar are so uncommon in West Africa as to warrant their being recorded.

A.H., age 23, was a missionary with the Sudan Interior Mission. He set out from Kano and completed a six months' trek, passing through the following places:—Zinder, Ingal, Tanout, Tahoua, Agades, Andaras, Iferuane, Dogondouchi, Dosso, Niamey, Say, Gaya, Llo, Yelwa, Jebba, and Minna. When he arrived at Minna he had fever, thought to be Dengue. It persisted for a few days and he was then sent to Kano and admitted to hospital. The pyrexia continued for the five weeks he was in hospital, and the cause remained obscure ; it did not respond to quinine.

He was then in a very weak state and was sent to England and admitted to the Liverpool School of Tropical Medicine. His temperature was 103° F. and pulse 120. He was extremely emaciated, cheeks very flushed, and had low muttering delirium from which he could be roused with difficulty. The spleen was palpable a hand's breadth below the costal margin.

A spleen puncture was performed and Leishman-Donovan bodies found in the material obtained.

The leucocyte counts are interesting :—

On admission   total leucocytes   ...   ... 625 per cmm.
5 days after admission   ,, ... 547 per cmm.
16 days after admission   ,, ... 937 per cmm.

Treatment with von Heyden 471 was commenced, but the patient died 14 March, 1928.

The diagnosis was by no means clear at first. A blood film showed a scanty infection with *Plasmodium malariae* and quinine was tried with but slight improvement. The Widal was negative, blood cultures were sterile, and agglutination tests for *Brucella melitensis* and *B. paramelitensis* were negative.

Dr. H. Peaston very kindly obtained a history of the case for me from Mr. E. F. Rice, who accompanied the patient on tour.

<div align="right">D. U. OWEN.</div>

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